TRANSPLANTATION
### Ciba Foundation Symposia

**General Volumes**

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* A leaflet giving details of the Ciba Foundation Colloquia on Endocrinology, Colloquia on Ageing and Study Group volumes is available from the Publishers
This volume is respectfully dedicated by the Chairman and members of the Symposium to the memory of the late
PETER ALFRED GORER, F.R.S.
1907–1961
The Ciba Foundation, a unique international institution, owes its inception to the generosity of CIBA Limited, Basle. However, being established under British trust law, it enjoys complete independence in practice and policy.

Under the guidance of its distinguished Trustees, the Foundation offers accommodation to scientists from all over the world at its home in Portland Place. Foremost in its activities is the organization of small conferences, the proceedings of which are published in book form in the manner of the present volume. The Foundation convenes many other informal discussions between research workers of different disciplines and different nationalities and each year invites an outstanding authority to deliver a special lecture. An exchange programme between French and British postgraduates is conducted and a library service is available. Furthermore, the Ciba Foundation attempts in every other way possible to aid scientists, whether they be Nobel Laureates or young graduates making their first original contribution to research.

The purpose of the Ciba Foundation, which is to promote international co-operation in medical and chemical research, is symbolized in the armorial bearings by five interlaced rings representing the continents, a black sacrificial cock (emblem of Aesculapius) holding a medical caduceus, and three regular hexagons for chemistry. Its domicile in London is indicated by the red sword of St. Paul and the British lion; the wyvern and the crozier, symbols associated with Basle, refer to the sponsoring firm located in this ancient Swiss town.
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PREFACE

The Ciba Foundation held a symposium on transplantation of tissues in 1953 and there have been many conferences elsewhere in recent years, notably in Paris in 1957 and in Liège in 1959. This subject's "take" is so vigorous that fresh material for a further international conference in London in 1961 would clearly soon be abundant, and Professor Medawar had already arranged this with the Director before his Award of the Nobel Prize set the seal on this project.

The Foundation itself suffered transplantation on this occasion; our own building being closed for reconstruction, we were fortunate to be accepted as a graft by the noble and ancient Society of Apothecaries, through the courtesy of its Master, Mr. A. M. A. Moore, and members of the Court. We are deeply indebted to them, and to the Society's staff. The graft was temporary, but we hope that the comparatively infant Foundation may prove that it is for ever tolerant to any call made upon it by our generous hosts of the Society.

The enthusiastic contributors to this volume, and its editors, hope that its publication will provoke a proliferative response in readers concerned with this complex and rapidly developing subject.
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List of those participating in or attending the Symposium on Transplantation held at the Worshipful Society of Apothecaries of London, 1st–3rd November, 1961

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R. E. Billingham . . . The Wistar Institute, Philadelphia
L. Brent . . . Dept. of Zoology, University College, London
J. M. Converse . . . Institute of Reconstructive Plastic Surgery, New York University School of Medicine, New York
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E. J. Eichwald . . . Laboratory for Experimental Medicine, Montana Deaconess Hospital, Great Falls
M. Feldman . . . Dept. of Experimental Biology, Weizmann Institute of Science, Rehovoth
M. Hašek . . . Biological Institute, Czechoslovak Academy of Sciences, Prague
W. H. Hildemann . . . Dept. of Infectious Diseases, University of California Medical Center, Los Angeles
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* Prevented from attending by illness.
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M. F. A. WOODRUFF . . Dept. of Surgical Science, University of Edinburgh
OPENING REMARKS

P. B. Medawar

Even though it makes a sad beginning to the Conference, our first thought on this occasion must be of the name that was missing when the Director read his roll call. Although Peter Gorer's discoveries and ideas will pervade all our discussions, now and for many years to come, we shall miss him grievously as a colleague and as a friend. Gorer had for many years been the world's leading authority on the serology and serological genetics of homograft reactions. He began his work in 1932, at University College, under J. B. S. Haldane; and there, using first a human serum and then a rabbit immune serum, he demonstrated isoantigenic variation in the red cells of mice. Very soon after he was able to demonstrate immune isoantibodies in mice, and in his classical papers from the Lister Institute in 1937 and 1938 he gave us all but conclusive proof of the immunological character of the reaction against homografts of tumours. His work attracted little attention, at all events in England; the war came, and his thoughts turned to other things; and it was not until after the war, in collaboration with his brilliant colleagues at Bar Harbor, that he began that detailed serological and genetical analysis of tissue transplantation in mice that underpins the entire theory of tissue transplantation. The more recent developments of his own and his pupils' work are very familiar to you: the demonstrations of passive immunity toward leukotic tumours, and of the vulnerability of lymphoid and myeloid cells to the action of humoral antibodies; the demonstration and titration of cytotoxins; the analysis of the mysterious "X-factor"; the work that has raised the possibility of a synergic co-operation between humoral antibodies and sensitized lymphoid cells. But
apart from these and other particular discoveries, we shall miss him above all for his general comprehension and grasp of all the problems of transplantation. With the Director’s warm approval, let us dedicate our present proceedings to the memory of Peter Gorer.

One of Gorer’s last works was to try to overhaul the terminology of transplantation research. We who study transplantation cannot acquit ourselves of the charge of making our ideas known to each other in a terminology that is etymologically ludicrous and inconsistent with certain older immunological usages. At the very least I suggest that we should follow Gorer (Table I) in substituting “allogeneic” for “homologous”, and in replacing “isologous” by “isogenic” or, much better, by “syngeneic”. Incidentally “enhancement” is another offender. It is a word to which Nathan Kaliss has given an exact meaning: “enhancement” is an abrogation of the homograft reaction mediated through the action of specific humoral antibodies. Where humoral antibodies are not known to be involved, should we not use a non-committal word like “promotion”—Flexner’s word, dating from 1907—instead?

At the last Ciba Foundation conference on transplantation, eight years ago, I began with a general review of transplantation theory of which the keynote was our incomprehension or ignorance of much that we should understand or know. No such review is called for today. We have in any event some reason to be satisfied with progress that has been made since we last met here. The serological analysis and genetic dissection of the H-2 locus I have already referred to. Here now, in no particular order, are some of the other accomplishments of the past eight years of research on homografts: the demonstration that, in many inbred strains of mice, male grafts are unacceptable to females—one of the most surprising single facts uncovered by the study of transplantation; the serological interpretation of “enhancement”; the discovery and analysis of “graft-versus-host” reactions, a discovery which
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has led to an astonishing proliferation of research, has provided a valuable new analytical tool, and has established important new principles in the field of general immunology, e.g. of the occurrence of immunologically competent cells in peripheral blood. (Here perhaps I should mention a cognate discovery, that lymphocytes, so far from being ephemeral, are long-lived cells that circulate and recirculate through the lymph nodes.) To continue, the past eight years have witnessed the extraction of sensitizing antigens from tissues and the development of a technique of assay, now being enlarged and reinforced by serological methods; the proof that the allogeneic—"homologous"—bone marrow cells used to repair radiation injury behave as grafts, and that a state having much in common with immunological tolerance may arise when irradiated mice are so treated; the use of transplantation techniques in the analysis of somatic cellular genetics, particularly of the variants that arise in populations of heterozygous tumour cells; the extension of the principles of transplantation immunity far down the ladder of vertebrate evolution, to amphibia and teleosts; the transfer of sensitivity to homografts in human beings by subcellular fractions of blood leucocytes; the revelation of the anomalous position of the golden hamster, and the analysis of why it should be so; the proof that two totally different tissues, skin epithelium and the endocrine component of the ovary, have a qualitatively similar representation of the histocompatibility antigens—with the important theoretical consequence that something between ten and fifteen neutral marker genes are present and at work in tissues which, though descended from the same zygote, have followed altogether separate pathways of differentiation; and the demonstration that in guinea pigs a delayed hypersensitivity reaction accompanies the rejection of homografts, a fact which strengthens the analogy between skin homograft reactions and the cell-mediated immunities. There is much else besides—for example, the slowly growing realization of the great differences between the reactivities associated with "strong" (in mice, with
H-2) and "weak" (non H-2) antigenic differences, accompanied by evidence that these weaker barriers of incompatibility are so much easier to break down than those erected by loci equivalent in strength to H-2. Nor should we forget the discovery of certain isolated phenomena which, though at present unexplained, may be looked back upon as being of crucial importance: the strange outcome of the intravenous injection of dissociated epidermal cells into rabbits; the privileged position of the hamster's cheek pouch; the anomalous behaviour, in guinea pigs, of thyroid homografts which have been allowed to reside for some months in the anterior chamber of the eye; the influence of an ovarian homograft on a later homograft of skin; and so on.

This is an inspiriting record, and one which workers in the field of transplantation research can take pride in. But—let us not forget that we are still quite ignorant of the proximate cause of the death of any homograft; the rôle of serum antibodies in transplantation immunity is still very far from certain; the chemical analysis of transplantation antigens is little more than embryonic; we are ignorant of much of the dynamics or kinetics of transplantation immunity, particularly as it concerns the origin and duration of sensitivity under different conditions; the relationship between tolerance and paralysis has yet to be laid bare; and, above all, we know of no harmless and lastingly effective way to subdue the homograft reaction in adult animals. We still have a very long way to go. In short, I think self-satisfaction and self-reproach should be about evenly balanced in our minds; and in that spirit let us begin this conference.
STUDIES ON TRANSPLANTATION ANTIGENS

L. Brent, P. B. Medawar and M. Ruszkiewicz

Department of Zoology, University College, London

Our work on transplantation antigens during the past two years has consisted of (1) an attempt to correlate the sensitizing power of antigens with their power to excite the formation of humoral antibodies in vivo or to inhibit their action in vitro; and (2) further investigations of the physical and chemical properties of cellular extracts containing antigenic matter.

The principle underlying the correlation referred to under heading (1) is straightforward. A graft transplanted from (for example) an A-strain donor to a CBA or C3H recipient has two distinguishable effects: it sensitizes its recipient, in the sense that a second graft from an A-strain donor will be destroyed more quickly than its forerunner; and it immunizes its recipient in the more conventional sense of provoking the formation of humoral antibodies. The isoantigens responsible for these two reactions have a common genetic determination, and the temptation is therefore to believe either that they are identical, or—as Snell (1957) has suggested—that they have similar determinant groups and differ only in respect of subsidiary attachments which affect the modality of the immune response. If either of these interpretations is true, then antigenic matter known to sensitize should also absorb (or, in soluble form, should inhibit the action of) the corresponding humoral antibodies.

The experiments of Hildemann and Medawar (1959) failed to uphold this interpretation. Their failure must be attributed to the inaccessibility of determinant sites in the very crude antigenic
preparations then (1957) in use. At all events, the antigenic preparation of Billingham, Brent and Medawar (1958) can absorb humoral antibodies in vitro and excite their formation in vivo (Brent, Medawar, and Ruszkiewicz, 1961). The preparation described by Herzenberg and Herzenberg (1961)—probably similar to, but anatomically better defined than our own—and the highly active preparation which Davies will describe shortly (see Davies and Hutchison, 1961) also absorb or inhibit the action of humoral antibodies; both have been subjected to scrupulous tests of specificity of action, and we await with great interest tests of their power to sensitize. Furthermore Lejeune and Kandutsch (this vol. p. 25 and 72) both make use of the specific serological activity of their sensitizing or “enhancing” extracts, and Stetson’s analysis (unpublished) of the anatomical distribution of iso-antigens within cells is founded upon their power to inhibit haemagglutination or the action of cytotoxins. The serological activity of tissue extracts with respect to the H-2 system of antigens in mice is therefore established beyond doubt. As our own work has just been published (Brent, Medawar and Ruszkiewicz, 1961) we shall not recapitulate it here.

Instead, we shall discuss three topics under the general heading of studies on the physical and chemical properties of extracts containing sensitizing antigens: (1) problems connected with the solubility or solubilization of antigens; (2) the behaviour of cellular extracts subjected to the action of lipid solvents; and (3) the heat-stability of sensitizing antigens.

In nearly all our experiments (exceptions are noted) we have used A-strain mice as donors of antigenic matter and of grafts, and CBA or C3H mice as their recipients. It is known of CBA mice (Barnes and Krohn, 1957), and can be assumed of C3H mice, that they differ from A-strain mice by antigens segregating at ten or more loci, but the serological and sensitizing activities we describe are dominated by “strong” antigenic differences at the H-2 locus.
Solubility and solubilization of sensitizing antigens

The solubilization of transplantation antigens is important not only as a prerequisite of some forms of physical and chemical analysis, but also because the sensitizing activity of antigens may be expected to vary with the forms in which they are presented to the responding subject.

It will be recollected that the preparation of Billingham and co-workers (1958) begins with the total disruption of A-strain lymphoid cells in distilled water, a process assisted by the exposure of the mechanically prepared homogenate to graded doses of ultrasound. Some old experiments of ours, of which only those with full internal controls are cited in Table I, show that these crude watery extracts contain some antigenic matter in a very finely subdivided form, and that its state of aggregation is profoundly influenced by the presence of electrolytes. If the crude aqueous extract is spun at 27,700 g (max.) for 30 to 45 min., sensitizing activity is about equally divided between sediment and supernatant fluid (Table I, A). If this supernatant fluid is now again spun for three or four hours at 134,000 to 173,000 g, it is still possible, mainly by the histological analysis of grafts, to discern a trace of sensitizing activity in the second supernatant (Table I, B). However, sensitizing matter behaves quite differently in the presence of electrolyte. If NaCl to a final concentration of 0.15M is added to the supernatant fluid after centrifugation at 27,700 g (procedure I, A), the first effect is the formation of a heavy precipitate of antigenically inert DNA-protein, and this may be removed by centrifugation at low speeds. After its removal the preparation is again spun at 27,700 g for 30 min.; most of the activity now appears in the sediment (Table I, C), and experience has shown that the sediment sensitizes more powerfully than the mother liquor from which it was derived. Evidently antigenic fragments tend to aggregate in the presence of electrolyte, and, as colloid theory would lead one to predict, the same consideration
Table I

Effect of electrolytes on solubility of antigenic matter prepared by ultrasonic dispersion of A-strain lymphoid cells in water

All experiments A→CBA except 201 and 287 (CBA→A). Sensitivity of recipients measured by degree of epithelial survival in A-strain body skin grafts removed 6 days after transplantation. Dose per mouse expressed in terms of original wet wt. in mg. of lymphoid tissue from which the antigen was extracted. Sec. u/s = time of exposure to ultrasound, in seconds.

[A] Original crude aqueous preparation before addition of electrolyte: roughly equal subdivision of activity between sediment and supernatant after spinning 30 to 45 min. at 27,700 g (max).

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<tr>
<th>Expt.</th>
<th>Dose</th>
<th>Sec. u/s</th>
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<th>Scores: supernatant</th>
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<td>30</td>
<td>25, 25, 25, 10, 5</td>
<td>75, 50, 25, 25, 10</td>
</tr>
</tbody>
</table>

[B] Supernatant resulting from procedure [A]: retention of perceptible fraction of sensitizing power by supernatant after further centrifugation at high speeds.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Sec. u/s</th>
<th>g (max.)</th>
<th>Spin, min.</th>
<th>Supernatant: Dose</th>
<th>Sediment: Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>227</td>
<td>60</td>
<td>134,000</td>
<td>180</td>
<td>320 100, 75, 50</td>
<td>360 50, 50, 0</td>
</tr>
<tr>
<td>237</td>
<td>30</td>
<td>173,000</td>
<td>240</td>
<td>450 100, 100, 100, 75</td>
<td>450 10, 10, 10, 0</td>
</tr>
<tr>
<td>251</td>
<td>45</td>
<td>173,000</td>
<td>240</td>
<td>360 90, 75, 75, 50</td>
<td>545 100, 75, 25, 5</td>
</tr>
<tr>
<td>262</td>
<td>45</td>
<td>173,000</td>
<td>240</td>
<td>430 75, 75, 50, 10</td>
<td>630 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

[C] Supernatant resulting from procedure [A]: after addition of NaCl to 0.15M the greater part of the activity appears in the sediment produced by a further centrifugation at 27,700 g for 30 min. Ultrasound: 30 sec. exposure throughout.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Dose</th>
<th>Scores: supernatant</th>
<th>Scores: sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>319</td>
<td>380</td>
<td>50, 50, 25, 25, 5</td>
<td>50, 10, 10, 0, 0</td>
</tr>
<tr>
<td>320</td>
<td>430</td>
<td>75, 50, 50, 25, 5</td>
<td>10, 5, 5, 0, 0</td>
</tr>
<tr>
<td>334</td>
<td>425</td>
<td>75, 50, 50, 50, 25, 25, 5, 0</td>
<td>25, 0, 0, 0, 0, 0</td>
</tr>
</tbody>
</table>

[D] Partition of activity between sediment and supernatant fluid when MgCl₂ or CaCl₂ (0·01M final conc.) is added to crude aqueous antigen, followed by centrifugation at ~2500 g for 10 min.

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Dose</th>
<th>Scores: supernatant</th>
<th>Scores: sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>275</td>
<td>50, 25, 5</td>
<td>50, 0, 0, 0</td>
</tr>
<tr>
<td>206</td>
<td>280</td>
<td>100, 75, 75, 75, 50</td>
<td>75, 25, 25, 25, 25, 5, 0</td>
</tr>
<tr>
<td>218</td>
<td>270</td>
<td>100, 75, 75, 75, 50, 25, 25, 5, 0</td>
<td>75, 50, 5, 0, 0, 0, 0,</td>
</tr>
</tbody>
</table>

Sec. Spin, Sec. Spin, th et, u/s = time of exposure to ultrasound, in seconds.
applies *a fortiori* when bivalent cations (e.g. CaCl₂ or MgCl₂ to a final concentration of only 0.01M) are added to the crude aqueous “solution”. Under these circumstances most of the antigenic activity appears in the sediment, accompanied by DNA-protein, after centrifugation at low speeds (Table I, D). We can take it that the antigenic matter contained in the original crude aqueous preparation consists of particles of very diverse sizes, and that even in the presence of 0.15M-NaCl some of the material is “soluble” in the sense defined by the above operations. The significance of these facts will be discussed later. The particles could well consist of fragments of cellular membranes, as Dr. Kandutsch has long insisted (Kandutsch and Reinert-Wenck, 1957; and see also Herzenberg and Herzenberg, 1961).

The “crude aqueous preparation” referred to in later experiments consists of an aqueous homogenate to which NaCl (final concentration 0.15M) has been added, and from which DNA-protein and undispersed matter has thereupon been removed by centrifugation at 5000 g for 10 min. The “antigenic sediment” with which the behaviour of this crude aqueous preparation will be compared is the sediment formed by a further centrifugation at 30,000 g for one hour. This sediment forms a typically “colloidal” suspension in water or physiological salt solutions, and it flocculates rapidly at pH 5.5 or less. The crude composition of the sediment is summarized in Table II. Its activity is such that, if a discriminating test is used, 0.25 mg, dry weight can be shown to sensitize a mouse; something like 0.5 mg. is needed to remove all detectable antibody from 1.0 ml. of a 1/50 dilution of a homologous antiserum of titre ~ 3200. These results do not encourage us to believe that the material is anything but grossly impure. On compositional grounds no inference can be drawn about the ingredient of the preparation in which activity lies; activity could reside in any one, or in any combination, of carbohydrate, lipid, or protein.

The solubilization of this insoluble sediment raises not one
problem, but two. For the isolation and purification of an active ingredient, much would be achieved if the preparation could be made to pass through a soluble stage, even if the matter finally used in biological tests were to be insoluble. It is therefore a

Table II

**Composition of antigenic sediment**

<table>
<thead>
<tr>
<th></th>
<th>Sugars (note 1)</th>
<th>Amino sugars (note 2)</th>
<th>Nitrogen (note 3)</th>
<th>Protein (note 4)</th>
<th>Lipid (note 5)</th>
<th>RNA (note 6)</th>
<th>DNA (note 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prep. 1</td>
<td>—</td>
<td>—</td>
<td>9.5</td>
<td>59.3</td>
<td>33.5</td>
<td>4</td>
<td>1.25</td>
</tr>
<tr>
<td>Prep. 2</td>
<td>2.25</td>
<td>0.85</td>
<td>9.4</td>
<td>58.7</td>
<td>34</td>
<td>4.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Prep. 3</td>
<td>2.3</td>
<td>0.93</td>
<td>8.5</td>
<td>53.1</td>
<td>34.5</td>
<td>3.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Prep. 4</td>
<td>—</td>
<td>—</td>
<td>9.3</td>
<td>58.1</td>
<td>36.7</td>
<td>40</td>
<td>—</td>
</tr>
</tbody>
</table>


(2) Neuhaus and Letzring’s modification (1957) of Elson-Morgan reaction; after hydrolysis with 2N-HCl for 3 hours. *Amino sugars identified*: glucosamine and galactosamine (approximately 1:1).

(3) Kjeldahl: Paul’s (1958) procedure, without fractionation.

(4) Total protein = nitrogen × 6.25.

(5) Fraction soluble in lipid solvents using Paul’s (1958) fractionation procedure; Bloor’s chromic acid method using recrystallized glycerol tripalmitate standard. *Sugars identified*: galactose, galactosamine, possibly trace of glucose. *Sugars identified in residues after lipid extraction*: galactose, mannose, glucosamine, trace of glucose and fucose, possibly trace of galactosamine. Mannose: galactose ratio approximately 1:1 (contrast note 1).

(6) Fraction soluble in perchloric acid (Paul, 1958); RNA estimated by Ceriotti’s (1955) orcinol reaction using ribose as standard.

(7) Fraction soluble in perchloric acid; DNA estimated by Ceriotti’s indole reaction (see Paul, 1958) using thymus nucleic acid (sodium salt) as standard.

matter of considerable importance that Kandutsch (1960 and in this volume) should have shown that his “enhancing” antigen can be made soluble without serious loss of potency in the non-ionic detergent Triton 100. (For all we know to the contrary, Kandutsch’s enhancing antigen might sensitize if administered in a way designed to reveal that capability.) In our experience, antigenic sediment which has been briefly exposed to 0.5 per cent
or 1.0 per cent Triton 100 or sodium deoxycholate no longer sensitizes upon injection. However, it is difficult to attach much weight to experiments in which the surface-active agent must either be injected in company with the antigen exposed to its action or be removed by organic solvents that may themselves be damaging.

For many biological purposes an antigen which has merely passed through a soluble state is not good enough: the antigen must be soluble in the form in which it is used. We have therefore taken advantage of Castermans' (1961) finding (cf. Mann, Corson, and Dammin, 1960) that sensitizing activity is not wholly lost by antigenic sediments which have been exposed to high pH's (in our experience, even as high as 11.0). We have found it convenient to bring an aqueous suspension of antigenic sediment to pH 10.0 or 11.0 by the very slow addition of 0.01N-NaOH, using an internal glass electrode system to monitor the pH, and preventing high local concentrations of alkali by brisk stirring with a plastic-coated magnetized iron bar. After removal of insoluble matter by spinning at 30,000 g for 30 min., the supernatant was brought to pH 8.0 with 0.01N-HCl, and NaCl was added to a final concentration of 0.15M. Any precipitate so formed was removed by a second centrifugation at 30,000 g for 30 min. The fraction of residual activity in the supernatant fluid has not yet been determined, but it is variable, and certainly very small.

The point of biological interest that has emerged from the use of this solubilized preparation, and of the original "crude aqueous preparation" (which contains some antigenic matter in a finely divided state), is that soluble or semi-soluble preparations do not sensitize when administered intravenously. This point may be taken as firmly established: the experiments summarized in Table III are confined to those that had strict internal controls. In addition there are faint indications, now being investigated, that an intravenous injection of soluble antigen may sometimes
Table III

INJECTION OF SOLUBLE (OR SEMI-SOLUBLE) ANTIGEN AND OF RESUSPENDED ANTIGENIC SEDIMENTS: COMPARISONS BETWEEN INTRA-VENOUS AND INTRAPERITONEAL ROUTES

All experiments A→C3H. Sensitivity tested by A-strain tail skin grafts transplanted 3 or 4 days after injection of antigen and either removed for histological examination 7 days later or scored by naked-eye inspection 11 days later. Dosages per mouse expressed in terms of mg. wet weight of A-strain lymphoid tissue from which the preparation started. Heparin, where used, 100 A.C.U. per mouse.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Nature of preparation</th>
<th>Heparin</th>
<th>Dose</th>
<th>Route</th>
<th>Graft survival scores:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>at 7 days</td>
</tr>
<tr>
<td>470A</td>
<td>Crude aqueous extract</td>
<td>present</td>
<td>250</td>
<td>I.V.</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>present</td>
<td>250</td>
<td>I.P.</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>absent</td>
<td>250</td>
<td>I.P.</td>
<td>—</td>
</tr>
<tr>
<td>471A</td>
<td>Resuspended sediment</td>
<td>present</td>
<td>250</td>
<td>I.V.</td>
<td>75, 50, 25, 10, 5</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>present</td>
<td>250</td>
<td>I.P.</td>
<td>50, 25, 5, 5, 0</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>absent</td>
<td>250</td>
<td>I.P.</td>
<td>5, 0, 0, 0, 0</td>
</tr>
<tr>
<td>D</td>
<td>Uninjected</td>
<td>absent</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>473A</td>
<td>Resuspended sediment</td>
<td>present</td>
<td>250</td>
<td>I.V.</td>
<td>50, 25, 25, 10, 0</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>present</td>
<td>250</td>
<td>I.P.</td>
<td>50, 5, 0, 0</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>absent</td>
<td>250</td>
<td>I.P.</td>
<td>25, 0, 0, 0, 0</td>
</tr>
<tr>
<td>D</td>
<td>Uninjected</td>
<td>absent</td>
<td>—</td>
<td>—</td>
<td>5 × 100</td>
</tr>
<tr>
<td>474A</td>
<td>Crude aqueous extract</td>
<td>present</td>
<td>250</td>
<td>I.V.</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>present</td>
<td>250</td>
<td>I.P.</td>
<td>10, 10, 5, 5, 0</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>absent</td>
<td>250</td>
<td>I.P.</td>
<td>5, 5, 0, 0, 0</td>
</tr>
<tr>
<td>475A</td>
<td>Solubilized at pH 11</td>
<td>absent</td>
<td>375</td>
<td>I.V.</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>absent</td>
<td>375</td>
<td>I.P.</td>
<td>75, 50, 25, 25, 5</td>
</tr>
</tbody>
</table>
prolong the life of homografts, but no firm reliance can be placed on this finding at present. Table III makes it clear that the insoluble antigenic sediment does sensitize when injected intravenously, though less effectively than via the intraperitoneal route; it also shows that the heparin (100 anticoagulating units per mouse) added as a precautionary measure to the material injected intravenously may itself weaken the sensitizing action of the antigen it accompanies.

Clearly an antigen which can be administered in a form and by a route which does not sensitize opens up new possibilities of subverting the homograft reaction, and for this reason we urgently await the preparation of a truly soluble antigen of high potency. It is tempting to think that our results may have some bearing on a phenomenon described by Billingham and Sparrow (1955)—the remarkable prolongation of the life of skin homografts in rabbits secured by the intravenous injection of living dissociated epidermal cells (see also Albert and Lejeune, 1959). Epidermal cells injected into the bloodstream presumably remain within the vessel walls, and might there liberate, in a soluble form, antigens which are known to be highly potent (Lejeune and Albert, 1960; Berrian and McKhann, 1960). However, two reservations should be borne in mind: (1) the absence of response to soluble “transplantation antigens” injected by the intravenous route might be a peculiarity of the mouse (cf. the work of Dresser, 1961), though there are certain reasons for thinking it may not be; and (2) the phenomenon may in any event be a trivial one, and depend merely upon the specially rapid degradation of antigen entering through the intravenous route, instead of along a pathway that gives direct access to lymph nodes.

**Extraction with lipid solvents**

The solubility and physical behaviour of sensitizing antigens are obviously affected by the high lipid content of existing prepara-
tions, and we have therefore examined the effect of lipid solvents on the sensitizing activity of dried antigenic sediment. Aqueous suspensions of antigenic sediments were shell-frozen at $-79^\circ$, "lyophilized" at a temperature not exceeding $-5^\circ$, and then further dried over phosphorus pentoxide at room temperature overnight under high vacuum (Brent, Medawar and Ruszkiewicz, 1961). From eight determinations, the weight of antigen dried under these conditions was $9.9 \pm 0.6$ mg. per gram wet weight of the lymphoid tissue from which the antigen was extracted. The dried antigen was extracted successively, at $0^\circ$ or $-15^\circ$, in $3:1$ v/v ethanol-ether, $3:1$ v/v ether-carbon tetrachloride, ethanol-ether as before, and ether. The solvents of each extraction were pooled, and the lipid emulsified in water either (1) by evaporating to dryness, redissolving in ethanol, adding water, and withdrawing alcohol on a rotary evaporator below room temperature; or (2) by adding water to the pooled extract and removing the organic solvents with a rotary evaporator as before—a simpler and more effective procedure.

The residue after exposure to lipid solvents was simply redispersed in normal saline, in which part of the protein now formed a clear solution.

The lipid fraction was tested for its sensitizing power, and the residue for its sensitizing power and its ability to absorb the corresponding haemagglutinins (see Brent, Medawar and Ruszkiewicz, 1961). The results of the tests in vivo are summarized in Table IV. The lipid fraction was inert, in the sense that the skin grafts used to disclose any state of sensitivity it might have produced enjoyed a normal expectation of life ($\sim 11$ days). The residue had no power to absorb humoral antibodies, but retained a small but clearly discernible fraction of its power to sensitize. Recombination of the lipid extract with the residue gave the same results as the use of the residue alone. These facts are commented upon at the end of the next section.
Table IV

Extraction of dried antigenic sediments with lipid solvents: activity of lipid extractives and of residues after extraction

All experiments A→C3H. Sensitivity tested by A-strain tail-skin grafts transplanted 3 or 4 days after the sensitizing injection and removed for scoring 7 days later.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Preparation</th>
<th>Dose* (mg.)</th>
<th>7-day survival score</th>
<th>Median survival times</th>
</tr>
</thead>
<tbody>
<tr>
<td>445B</td>
<td>Residue after extraction</td>
<td>3·3</td>
<td>75, 10, 5, 0, 0</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>Lipid extractive</td>
<td>3·3</td>
<td>(100, 100, 100, 100, 100)</td>
<td>11 days</td>
</tr>
<tr>
<td>D</td>
<td>Mixture of B &amp; C</td>
<td>3·3</td>
<td>75, 50, 0, 0, 0</td>
<td>—</td>
</tr>
<tr>
<td>446B</td>
<td>Residue after extraction</td>
<td>3·5</td>
<td>90, 50, 10, 10, 0</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>Lipid extractive</td>
<td>3·5</td>
<td>(100, 100, 100, 100, 100)</td>
<td>10 ½ days</td>
</tr>
<tr>
<td>D</td>
<td>Mixture of B &amp; C</td>
<td>3·5</td>
<td>50, 50, 50, 5, 0</td>
<td>—</td>
</tr>
<tr>
<td>E</td>
<td>Nil (control grafts)</td>
<td>—</td>
<td>(100, 100, 100, 100, 100)</td>
<td>11 days</td>
</tr>
<tr>
<td>449D</td>
<td>Residue after extraction</td>
<td>3·8</td>
<td>75, 75, 75, 50, 25</td>
<td>—</td>
</tr>
<tr>
<td>E</td>
<td>As D but soluble matter†</td>
<td>3·8</td>
<td>100, 100, 100, 100, 75</td>
<td>—</td>
</tr>
<tr>
<td>F</td>
<td>As D but insoluble matter†</td>
<td>3·8</td>
<td>75, 75, 75, 50, 25</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>Lipid extractive</td>
<td>4·9</td>
<td>(100, 100, 100, 100)</td>
<td>10 ½ days</td>
</tr>
<tr>
<td>E</td>
<td>Nil (control grafts)</td>
<td>—</td>
<td>(100, 100, 100, 100, 100)</td>
<td>11 days</td>
</tr>
<tr>
<td>451B</td>
<td>Untreated P₂O₅-dried antigen</td>
<td>2·5</td>
<td>5, 5, 5, 5, 0</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>Residue after extraction</td>
<td>4·5</td>
<td>75, 75, 50, 25, 10</td>
<td>—</td>
</tr>
<tr>
<td>D</td>
<td>Lipid extractive</td>
<td>4·5</td>
<td>100, 100, 100, 100, 100</td>
<td>—</td>
</tr>
</tbody>
</table>

* In terms of mg. dry weight P₂O₅-dried antigen per mouse.
† After centrifugation at 5000 g for 10 minutes.

Heat stability

A most unexpected result of our experiments on the thermostability of sensitizing antigens was evidence that some fraction of the sensitizing power of antigenic sediments survived exposure to 100° for from four to 12 min. The sediment was redispersed in gas-free water and heated under an atmosphere of nitrogen in a boiling water bath. As with the residues of lipid extraction, antigen so treated had no power to absorb the homologous iso-agglutinins, but it retained a certain low but well-defined sensitizing power (Table V), predominantly in the insoluble fraction.
<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Experimental treatments</th>
<th>7-day survival scores</th>
<th>Control treatment</th>
<th>7-day survival scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>439</td>
<td>100°, 4 min.</td>
<td>25, 5, 0, 0, 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>441A B</td>
<td>100°, 4 min.</td>
<td>50, 5, 5, 0, 0</td>
<td>Untreated mice</td>
<td>5 × 100</td>
</tr>
<tr>
<td></td>
<td>100°, 12 min.</td>
<td>75, 50, 25, 0, 0</td>
<td>C3H Ag, 100°, 4 min.*</td>
<td>4 × 100</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td>C3H Ag, unheated*</td>
<td>5 × 100</td>
</tr>
<tr>
<td>447A B</td>
<td></td>
<td></td>
<td>C3H Ag, 100°, 4 min.*</td>
<td>4 × 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C3H Ag, unheated*</td>
<td>5 × 100</td>
</tr>
<tr>
<td>448A B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>450A B</td>
<td>Total preparation</td>
<td>90, 75, 50, 10, 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Supernatant only†</td>
<td>75, 75, 75, 50, 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Sediment only†</td>
<td>75, 50, 25, 10, 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>As A, trypsinized‡</td>
<td>5 × 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td>C3H Ag, 100°, 5 min.*</td>
<td>5 × 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Untreated mice</td>
<td>4 × 100, 90</td>
</tr>
<tr>
<td>452B C</td>
<td>Total preparation</td>
<td>75, 50, 50, 50, 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Supernatant only†</td>
<td>75, 75, 50, 50, 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Sediment only†</td>
<td>50, 25, 0, 0, 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>As B, trypsinized‡</td>
<td>5 × 100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* C3H mice treated with heated or unheated C3H (isogeneic) "antigen" (Ag).  † After centrifugation at 30,000 g for 30 min.  ‡ "Total" preparation exposed to trypsin (0.5 mg. per mouse dose) for 15 to 30 min. at room temperature, pH 7.4.
In two experiments, antigenicity was abolished by tryptic digestion for 15 to 30 min. at room temperature. It will be seen from Table V that the injection of heated C3H “antigen” into C3H mice had no effect on the survival time of homografts, a result which seems to rule out a non-specific activation of the host’s response.*

The upshot of the experiments described in the last two sections is that antigenic matter lacking in detectable serological activity may yet sensitize against homografts in vivo. It is not yet possible to distinguish between the following explanations of this fact: (1) the residual sensitizing activity belongs to an antigen of the H-2 complex, but the combining sites necessary for the absorption or inhibition of antibody in vitro are rendered inaccessible by physical changes accompanying denaturation; or (2) there is a genuine difference between “T” forms and “H” forms of iso-antigens (in the terminology of Medawar (1959)), the former being the more stable. This second interpretation now carries little conviction. A third possibility, (3), is that the many antigens by which strain A differs from strain C3H—antigens governed by loci other than H-2—are a chemically heterogeneous assemblage (cf. Berrian and Jacobs, 1959), and that some are thermostable or resistant to lipid solvents. This seems at present to be the most plausible explanation; the chief obstacle in the way of accepting it is that we should not expect these weaker antigens to be revealed by a test in which the skin graft used to measure the degree of sensitivity is transplanted as soon as three days after the sensitizing injection.

Summary

It is shown that the physical form and route of ingress of sensitizing cellular extracts affect their immunological perfor-

* The injection of “antigen” of syngeneic (isologous) origin is the most convincing test of specificity of action when a complex of sensitizing antigens of different genetic determination is distributed in an unknown manner among the various inbred strains of mice.
STUDIES ON TRANSPLANTATION ANTIGENS

Some small fraction of the sensitizing activity of cellular extracts survives (1) their exposure to lipid solvents, and (2) heating to 100°. (The matter extracted into lipid solvents did not sensitize.) Antigens treated in this way did not absorb the homologous humoral antibodies. One possible explanation of this fact is that the antigens which differentiate strain A from strains C3H or CBA are a chemically heterogeneous assemblage.

Acknowledgement

The special expenses of this work and the stipend of one of us (M.R.) were defrayed by grant E-3137 of the United States Public Health Service.
DISCUSSION

Silvers: I wonder if it would not be possible to differentiate the H-2 factors from the other factors by doing similar experiments with co-isogenic resistant mice, where you would only be dealing with a single H-2 difference.

Medawar: Ideally, of course, one should use co-isogenic lines. Unfortunately we suffer from a chronic shortage of mice and it would be a grave logistical problem to change over from our domestic strains to co-isogenic strains.

Silvers: Because in the mouse there is apparently only one histocompatibility locus, the H-2, which is concerned with the production of strong transplantation antigens, it might be advantageous to work with other species. For example, we are maintaining two inbred strains of rat which differ by at least 16 histocompatibility factors. Moreover, four of these loci are concerned with the production of transplantation antigens which can elicit as strong an immunological response as the H-2 antigens of the mouse. By utilizing these rat strains it is conceivable that one might be able to define, in biochemical terms, the transplantation antigens controlled by genes at more than one locus. This would probably be difficult to accomplish with the present methods in the mouse.

Medawar: This would indeed be worth studying. However, there may be other strong histocompatibility loci in mice which have not yet been identified.

Woodruff: On the question of the route of injection—this seems to me very relevant to the differences in behaviour of organs transplanted by vascular anastomosis and free grafts. There is presumably a component of lymphatic and a component of vascular absorption in each case, but the timing is different in the two. With organ grafts, local irradiation may disturb the balance and perhaps reduce the lymphatic components, partly by interfering with the regional lymph nodes and partly by a direct strangulating effect on the lymphatic vessels.
Medawar: The possibility of antigens entering the recipient through the intravenous route is something we could perhaps keep in mind when thinking not only of whole organ grafts, established by vascular anastomoses, but also of the relations between the foetus and the mother, between which the traffic is presumably intravenous. As to the inefficiency of the intravenous route generally, there is evidence that this is not a peculiarity of the mouse—it was first described in the rabbit, and there is some evidence also from human beings; intravenous infusions of living whole blood in human beings, according to Friedman and his colleagues in Boston (Merrill, J. P., Friedman, E. A., Wilson, R. E. and Marshall, D. C. [1961]. J. clin. Invest., 40, 631) do not sensitize, whereas intradermal injections of leucocytes quite certainly do.

Brent: To complete the catalogue of the situations in which the intravenous route behaves anomalously, there are the experiments of F. Shapiro and his colleagues. They find that if they inject vast numbers of allogeneic cells \(1.5 \times 10^9\) intravenously into adult animals, they can make the animals unresponsive; but the intraperitoneal injection of a similar number of cells apparently does not lead to unresponsiveness.

Eichwald: How well satisfied are you with your method of assaying survival of skin grafts in terms of percentage?

Medawar: It is not very good because it is a subjective and rather empirical process. However, Brent and I don't disagree by very much when we score grafts independently.

Woodruff: Do you think it is likely to be a general rule that the significant sensitizing antigens in species other than the mouse are the particular ones which also stimulate the formation of haemagglutinins? In other words, is there a human or rat or rabbit or dog locus with similar properties to the H-2 locus of the mouse? It seems to me that there is no reason for assuming it at all.

Barrett: My experience suggests caution in generalizing about these antigens. Some of this emphasis on the H-2 locus is certainly justified on the basis of our information in the mouse. But in my own laboratory and with my own materials, the properties of the blood (injected from one strain of mouse into another) that will induce dextran-type haemagglutinins and on the other hand those other properties that will produce resistance either against skin grafts or against the implantation
of tumour, are wholly dissociable except genetically; they are not alike in their sensitivity to temperature, to formalin, to ordinary forms of lysis, or to high-pitched audible, not supersonic, sound. Generalization here may be very misleading.

Medawar: I entirely agree, one must always keep in mind the particular strain combination one is dealing with. One cannot generalize, for example, from H-2 to non-H-2 differences in mice.

Barrett: In the mouse and some other species we emphasize the importance of these loci which seem to control the generation of haemagglutinins. I am not too familiar with the literature on this but I believe that plastic surgeons have found that blood grouping in the human being is not informative as to the outcome of subsequent grafts. I would like to hear the views of members of the symposium on this.

Medawar: All that has been proved about human blood groups and their relations with grafts is that blood-group compatibility is not sufficient for the success of grafts; but it may well be necessary.

Brent: Having recently had an opportunity of reading the literature on kidney transplantation in human beings, it struck me very forcibly that in the four cases in which there has been moderately successful transplantation of kidneys with the aid of irradiation there has also been pretty good correlation between the red cell antigens of the donor and recipient. This rather suggests that matching of red cell antigens can be a rough guide as to the chances of success in organ transplantations.

Kandutsch: I am not a serologist, but it seems to me that one cannot obtain any absolute measure of the purity of an antigenic preparation from the absorption of antibodies. Dr. Stimpfling has done this test with our preparations but I have not thought that it was possible to judge purity on this basis.

I would like to hear more about the inactivating effect of Triton on your preparation. We have used this extraction with Triton for several years now, and we get very little inactivation. I wonder if you could tell me in more detail the pH and other conditions under which you carried out this inactivation.

Medawar: The difficulty about our in vivo experiments with deoxycholate and Triton is that unfortunately we have to inject the whole preparation, detergent and all, and the antigen is thereupon promptly precipitated. The pH was neutral and the material was injected after it
had been centrifuged (not at very high speeds, but so that it was free of crude material not solubilized by deoxycholate or Triton). We have also tried Tween 80 and this did not solubilize nor did it greatly impair sensitizing activity. Therefore with these three surface-active agents inactivation and solubilization went hand in hand. Although I find it fundamentally objectionable to inject antigenic preparations containing these surface-active substances, yet when we tried your method of removing the Triton with acetone, reprecipitating the antigen, the preparation was totally inactive in our system.

_Hildemann:_ I would like to raise two questions relative to your finding that soluble extracts would not sensitize by the intravenous route. Will such extracts under any conditions lead to prolonged homograft survival, as you supposed at one point in your presentation? Secondly, will such intravenously administered extracts induce humoral antibodies and, if so, how does the titre compare with the antibodies induced by, say, the intradermal route, which gives you effective transplantation immunity as well?

_Medawar:_ In answer to your first question: we have isolated observations of a prolongation of the life of homografts by injecting these materials intravenously; but I emphasize that these are just isolated observations.

As to whether these extracts provoke humoral antibodies when injected intravenously: we have injected only four mice repeatedly with these semi-soluble extracts intravenously. Three out of four of them produced no haemagglutinins and no haemolysins and one of them produced a moderate titre of both—so we don’t know what to make of it.

_Amos:_ Haemagglutination is a legitimate indicator in the mouse as long as you are certain you are dealing with H-2, but one must bear in mind that there are a number of haemagglutinating systems not related to H-2 (we have already identified about seven of these) and there are other cases where the antigen is not on the red cell (H-1 and H-3 are two good examples of this). If you now want a quantitative estimate of the antibody response you must have some idea whether there is a contamination with some of these other antibodies; this should not be difficult to determine using back-cross animals as indicators. Silvers’ suggestion of using the co-isogenic lines is an excellent
one. You certainly can’t extrapolate from the mouse to any other species; this coincidence of histocompatibility antigens on the red cell and on the other tissue cells is purely fortuitous.

*Woodruff:* Is it fortuitous? Is there any evidence that this is purely a mouse phenomenon?

*Amos:* I don’t know if I can answer that completely. Dr. Billingham, in the rat, how many of these other systems also show up with haemagglutinating antibodies?

*Billingham:* From our analyses with the unrelated B.N. and Lewis rat strains we know that there are at least 16 different histocompatibility loci in this species, of which 4 appear to be of major status (Billingham, R. E., Hodge, B. A., and Silvers, W. K. [1962]. *Proc. nat. Acad. Sci. (Wash.),* in press). Dr. Joy Palm has recently reviewed the current status of knowledge of blood groups in the rat ("Blood Groups in Infra-Human Species," in *Ann. N.Y. Acad. Sci.*, 1962, in press). In her own studies with the Lewis and B.N. strains she has found that genes of at least one blood group locus are important in determining the incompatibility of skin homografts.

*G. Klein:* Dr. Amos, is your statement that the coincidence of transplantation antigens on the red cells and on other tissue cells is purely fortuitous, based mainly on the fact of haemagglutination? Could it be that most antigens are in fact present on red cells but some of them are unable to cause haemagglutinability?

*Amos:* I don’t think so; this is a quantitative question. I can answer this in an indirect way in terms of absorption, that there is a completely different pattern of absorption obtained with some of the non-H-2 antibodies. Certainly the quantitative differences in the amounts of antigens are quite different with respect to different antigens. Within the H-2 system the distribution of antigens appears to be much the same, so that for any given organ, no matter whether it is H-2$^a$, H-2$^b$, H-2$^d$, etc. the level as judged by absorptive capacity is comparable. But if you now move to a system other than H-2, the relative proportion of antigens in different tissues will be quite different, e.g. alpha, which is in high concentration in kidney while H-2 is low in this organ. I think this relationship is different again for H-1 and H-3. This is why I think it is rather fortuitous. It is a question of the activity of the genes controlling the particular tissue that you’re dealing with.
TRANSPLANTATION ANTIGENS, PRODUCTION OF HAEMAGGLUTININS AND INHIBITION OF THE HAEMAGGLUTINATION REACTION

G. Lejeune-Ledant*

Laboratoire de Chirurgie Expérimentale, Université de Liège

All the experimental work performed in recent years indicates that transplantation of living tissues or injection of living cells elicits both transplantation immunity and humoral immunity (Medawar, 1959).

The humoral immunity can be demonstrated by the appearance of isoantibodies and especially haemagglutinins (Gorer and Mikulska, 1954; Amos et al., 1954). Of course, many other humoral antibodies may be identified, for instance haemolysins (Hildemann, 1957) leuco-agglutinins (Amos, 1953), cytotoxins (Gorer and O’Gorman, 1956), but the haemagglutination reaction is perhaps the easiest and the most accurate one, principally since Gorer (1947), in his important pioneer work in this field, devised his method employing dextran and human serum.

Transplantation immunity can easily be identified by the biological test of the second-set phenomenon (Billingham, Brent and Medawar, 1956). If living tissue grafts elicit both a transplantation immunity and humoral immunity, two questions should be answered:

1. Does any antigen—whatever its form may be, living cells or cell-free extracts—which sensitizes to a skin homograft of the same donor provoke the formation of haemagglutinins?

2. If so, should not any antigen of these various kinds which

* Dr. Lejeune-Ledant was not able to attend the meeting due to illness, and his paper was presented by Prof. F. Albert.
cause transplantation immunity be capable of absorbing those haemagglutinins, in this way producing an inhibition of haemagglutination reactions? If this question could be cleared up, haemagglutination inhibition would become a simple and rapid test for the detection of any transplantation antigen. The importance of such a test need hardly be emphasized. Indeed, the present ways of identifying transplantation antigens consist in biological techniques, such as induction of transplantation tolerance (Billingham, Brent and Medawar, 1953), production of transplantation immunity (Billingham, Brent and Medawar, 1956), or production of cutaneous hypersensitivity (Brent, Brown and Medawar, 1958). Everybody knows, first, that the induction of tolerance by cell-free extracts has not been very effective so far, and secondly that the second-set response and the cutaneous hypersensitivity reaction are time-consuming tests and, at least as far as the last one is concerned, at the mercy of non-specific influences (Medawar, 1959).

The aim of the following group of experiments has been to verify the presence or absence of haemagglutinins in mice after administration of different types of transplantation antigens.

A. Production of haemagglutinins after administration of various transplantation antigens

The mice used in this group of experiments are all of the lines CBA and SA, the CBA used as donors, the SA as recipients. If the H-2 antigenic combination in CBA mice is relatively well known, we cannot say the same for the SA line. In our experimental conditions, the normal survival time of a skin homograft from CBA mice to SA mice is $10.5 \pm 0.6$ days.

Preparation of transplantation antigens

Living spleen cells, epidermal cells and cell-free extracts of various kinds have been used. All the antigenic material is injected by the intraperitoneal route, except for the epidermal cells.
The spleens collected, with all required aseptic precautions, are gently chopped and expressed through a stainless steel sieve into buffered normal saline at 0°C. The cells isolated by vigorous pipetting are counted, disregarding the red cells.

Spleenic extracts are prepared by two different techniques:

1. the technique of Billingham, Brent and Medawar (1958), where the cells are brought into solution in water by a short exposure to ultrasonic irradiation, DNA proteins being precipitated by the addition of NaCl to 0.15-M strength and removed by centrifugation at 2,000 g. The supernatant is finally spun at 25,000 g for one hour.

2. the technique of Oth and Castermans (1959) by which the extraction is obtained by homogenization in a Waring blender in an ice-cold solution of 0.14 M-NaCl, 0.001-M sodium citrate, and a secondary acid precipitation at pH 5.5.

Epidermal cells are prepared according to a previously described method (Albert and Lejeune-Ledant, 1959). Skin taken from the tails of the mice is collected in a buffered solution and submitted to the action of trypsin for 20 minutes at 37°C. The epidermal sheets are expressed through the stainless steel sieve, and the cells isolated by pipetting. These epidermal cells are always injected intravenously, the only route we have found effective for induction of a transplantation immunity (Lejeune-Ledant, 1960).

Epidermal extracts (Lejeune-Ledant and Albert, 1960) are obtained by digestion of the isolated cells submitted to the action of a 0.25 mg. per cent concentration of trypsin in a buffered solution: 0.137 M-NaCl, 0.0026 M-KCl, 0.0081 M-Na2HPO4, 0.00147 M-KH2PO4, 0.0009 M-CaCl2 and 0.00048 M-MgCl2·6H2O. Ca and Mg ions are intended to protect the antigens against the action of trypsin. After incubating from 5 to 20 minutes at 37°C, all the subsequent manipulations are performed at +4°C. Trypsin is eliminated by repeated washings in 0.15 M-NaCl and centrifugation at 1500 g. The final antigenic extract is suspended in pure water.
Serological method

Antisera have been titrated principally by the haemagglutination technique (with slight modifications) in the presence of dextran and absorbed human serum (Gorer and Mikulska, 1954). Only these results are reported in this paper.

The tubes used for this test are 7.5 mm. in diameter and 65.7 mm. long. Reagents are mixed in the ratio of 0.05 ml. antiserum in doubling dilution in 1.8 per cent dextran (Glaxo Intradex 10 per cent, salt-free in 5 per cent glucose) to 0.05 ml. of a 2 per cent suspension of CBA erythrocytes in 50 per cent v/v absorbed human serum. The tubes are incubated for 90 minutes at 37° and centrifuged at 600 g for 30 seconds.

The degree of agglutination is observed macroscopically, using gentle agitation, and is verified microscopically, particularly to establish end points. Two control tubes are regularly used: in the first, antiserum is replaced by 1.8 per cent dextran; in the second, absorbed human serum is added. The specificity of the reaction is tested with isologous red cells.

Results

Injection of living cells. As shown in Table I, high titres of haemagglutininins are only produced by repeated injections of living cells—either spleen cells or epidermal cells. In our experimental conditions, a single injection of living spleen or epidermal cells, although evoking transplantation immunity, is unable to elicit a detectable titre of haemagglutininins. Specificity of the reaction is tested with isologous SA red cells, which give unvaryingly negative results.

Injection of cell-free extracts. The cell-free extracts are injected at a concentration which will induce a violent transplantation immunity in a control animal, with an accelerated breakdown of the skin graft. The amount of splenic extracts used corresponds to $250 \times 10^6$ spleen cells, and the amount of epidermal extracts, which are much more active, to $20 \times 10^6$ epidermal cells. These
Table I

**Antibody titres obtained by the injection of CBA cells into SA mice**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>No. of recipients</th>
<th>Dosage of cells per injection per mouse ($\times 10^6$)</th>
<th>Interval of injections in days</th>
<th>Serum taken on days after last injection</th>
<th>Red-cell antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>Spleen cells (intraperitoneal)</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>70 - 36</td>
<td>7</td>
<td>10</td>
<td>1/64</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>75 - 14 - 40</td>
<td>7</td>
<td>10</td>
<td>1/256</td>
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<tr>
<td>4</td>
<td>10</td>
<td>100 - 150 - 150 - 140</td>
<td>7</td>
<td>10</td>
<td>1/512</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>200 - 250 - 250 - 250</td>
<td>7</td>
<td>10</td>
<td>1/1024</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>Epidermal cells (intravenous)</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>2 - 2</td>
<td>7</td>
<td>10</td>
<td>1/16</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>2 - 2 - 2</td>
<td>7</td>
<td>10</td>
<td>1/32</td>
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<tr>
<td>9</td>
<td>10</td>
<td>1 - 1 - 1 - 1</td>
<td>7</td>
<td>10</td>
<td>1/64</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>1 - 1 - 1 - 1 - 1</td>
<td>7</td>
<td>10</td>
<td>1/128</td>
</tr>
</tbody>
</table>

Table II

**Antibody titres obtained by the injection of CBA cell-free antigenic extracts into SA mice**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>No. of recipients</th>
<th>Dosage of cell-free extracts per injection (intraperitoneal) ($\times 10^6$)</th>
<th>Interval of injections in days</th>
<th>Red-cell antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>9</td>
<td>Spleen cells: 250 (BBM)</td>
<td>0 - 3 - 9 - 19 - 23</td>
<td>1/128</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>Spleen cells: 250 (OC)</td>
<td>0 - 3 - 9 - 19 - 23</td>
<td>1/64</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>Epidermal cells: 20 (LA)</td>
<td>0 - 3 - 9 - 19 - 23</td>
<td>1/128</td>
</tr>
</tbody>
</table>

BBM: Extracts prepared by technique of Bellingham, Brent and Medawar (1958).
LA: Extracts prepared by technique of Lejeune-Ledant and Albert (1960).

extracts are injected by the intraperitoneal route in groups of 10 recipients at multiple intervals. The titre of haemagglutinins is checked in the pooled serum of all the recipients of the same group. The specificity is again verified with isologous red cells. As shown by the results given in Table II, all the types of cell-free extracts elicit the formation of haemagglutinins.
In conclusion, all parts of these experiments demonstrate that all the transplantation antigens—both living cells and cell-free extracts—will elicit the formation of haemagglutinins in the blood of recipients, provided that a sufficient antigenic stimulus is given.

B. Inhibition of the haemagglutination reaction by transplantation antigens

Since the previous experiments have demonstrated that all the transplantation antigens tested lead to the formation of serum antibodies (recognized by their ability to agglutinate the red cells of the donor) it seemed logical to assume that the same transplantation antigens should be able to absorb these agglutinins, and consequently inhibit the haemagglutination reaction.

We (Albert and Lejeune-Ledant, 1959) were specially interested by the suggestion that transplantation antigens belong to the category of "mucoids" and perhaps are very similar to the blood group substances (Billingham, Brent and Medawar, 1958). These authors pointed out that transplantation antigens are inactivated by *Trichomonas foetus* enzymic action and by periodate, both well known to inactivate the blood group substances. These blood group substances are detected and selected by the haemagglutination inhibition test.

The use of extracts prepared by the various techniques used for the isolation of blood group substances seemed to be specially attractive. All these extracts are soluble—a particularly favourable condition for the study of the chemical constitution of transplantation antigens. Results of these experiments have been published elsewhere (Albert and Lejeune-Ledant, 1959). Neither crude gastric extracts prepared according to the technique of Friedenreich and Hartmann (1938) nor more highly purified extracts prepared with the phenol alcohol technique of Kabat (1956), injected into recipients of another strain of mice, ever produced a transplantation immunity. On the contrary, when
the recipients were challenged with a skin homograft of the same donor, they showed regularly an actual prolongation of the survival of their grafts. This phenomenon is not, however, entirely specific and may be obtained in some cases with isologous extracts. It seemed worth while to test in parallel the action of various cell-free extracts, including the latest ones, on the haemagglutination reaction.

**Haemagglutination inhibition method**

Hyperimmune serum SA anti-CBA is diluted in dextran solution at $1.8$ per cent in three series of tubes. In all the series, the first two tubes are used as controls and contain only a $2$ per cent red cell suspension and the dextran solution. In the other tubes of each series, hyperimmune serum is diluted at various concentrations: $1/1$, $1/40$, $1/100$, $1/200$, $1/1000$.

The first series gives the titre of the haemagglutinins.

The second series contains the hyperimmune serum SA anti-CBA and the homologous extract CBA in contact for 30 minutes at $4^\circ$. After this incubation, red cell suspensions in $50$ per cent v/v absorbed human serum are added. The haemagglutination reaction is read after 60 minutes' incubation at $37^\circ$.

The third series of tubes is prepared in the same manner, but contains the isologous SA extracts.

For the reaction to be specific, inhibition of the haemagglutination must be positive only with the homologous CBA extract.

**Results**

Our results are summarized in the Tables III and IV.

All cell-free extracts, whatever the kind of extraction technique, which cause the production of transplantation immunity in the recipient, detected by a second-set phenomenon when challenged with a skin homograft of the same donor, all such extracts produce a specific inhibition of the haemagglutination reaction. Indeed, there is no inhibition when isologous extracts are used.
On the other hand, all the extracts prepared according to the methods of preparation of the blood group substances, which, instead of producing a transplantation immunity, give a prolonged survival of the homografts of the same donor, all these extracts may be able to inhibit the haemagglutination reaction. However, both these phenomena show a total lack of specificity.

Table III

<table>
<thead>
<tr>
<th>Serum</th>
<th>Control</th>
<th>1/1</th>
<th>1/40</th>
<th>1/100</th>
<th>1/200</th>
<th>1/1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. SA anti-CBA</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>II. SA anti-CBA + 2 mg. epidermal extract CBA*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III. SA anti-CBA + 2 mg. epidermal extract SA*</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* In wet weight and prepared by technique of Lejeune-Ledant and Albert (1960).

Table IV

<table>
<thead>
<tr>
<th>Serum</th>
<th>Control</th>
<th>1/1</th>
<th>1/40</th>
<th>1/100</th>
<th>1/200</th>
<th>1/1000</th>
</tr>
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<tr>
<td>I. SA anti-CBA</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>II. SA anti-CBA + 2 mg. splenic extracts CBA (BBM)*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>III. SA anti-CBA + 2 mg. splenic extracts SA (BBM)*</td>
<td>—</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* In wet weight and prepared by technique of Billingham, Brent and Medawar (1958) (BBM).
† In wet weight and prepared by technique of Oth and Castermans (1959) (OC).
Discussion

When we started this experimental work, we were trying to answer the two questions:

(1) Does antigen sensitizing to a skin homograft of the same donor elicit the formation of haemagglutinins?

(2) Is an antigen of this kind capable of producing an inhibition of the haemagglutination reaction?

As far as the first point is concerned, the problem was far from being solved. Gorer had stressed many times the fact that the A strain is specially favourable for this kind of work. However, Hildemann and Medawar (1959) recently pointed out that if the combination A donor and CBA recipient was indeed serologically the most favourable, the reverse combination CBA donor and A recipient was much less favourable and did not give regularly detectable haemagglutinins.

A long series of experiments, briefly reported in this paper, demonstrates first that all living cells (especially spleen and epidermal cells) capable of inducing transplantation immunity, when injected several times always elicit the appearance of haemagglutinins in the blood of the recipient.

Once this point was clearly demonstrated for the combination of strains we used, it became much more interesting to verify if the cell-free extracts of those cells had the same properties.

Once again, the answer is yes, provided that a sufficient antigenic stimulus is given by repeated injections; the cell-free extracts too elicit the formation of haemagglutinins in the blood of the recipient. The fact that all the antigens—living cells or cell-free extracts—are capable of producing at the same time humoral immunity and transplantation immunity suggests that there must be a very close similarity between antigens forming humoral antibodies and transplantation antigens.

As far as the second point is concerned, the question is of practical importance: if all the transplantation antigens are
actually able to inhibit specifically the agglutination reaction, this inhibition gives us a simple and rapid test for the detection of any transplantation antigen.

Our experiments show that any antigen which causes a transplantation immunity identified by the second-set phenomenon, specifically inhibits the haemagglutination reaction. On the other hand, all the cell-free extracts prepared with the technique used for the preparation of blood group substances, which do not cause a transplantation immunity but a prolonged survival of skin homografts of the same donor, inhibit, non-specifically, the haemagglutination reaction. All our complementary assays agree that all the cell-free extracts producing an inhibition of the haemagglutination reaction, and only these extracts, are able to elicit a transplantation immunity and thus may be considered as transplantation antigens.

Conclusions

All homologous cell-free extracts able to induce transplantation immunity also elicit a humoral immunity identified by the formation of haemagglutinins.

The same extracts inhibit the haemagglutination reaction specifically.

The results from all our completed experiments and from others now in progress agree that the inhibition of the haemagglutination reaction appears to be an accurate and rapid test for the detection of any transplantation antigen.

Summary

In mice, homologous isolated cells such as spleen or epidermal cells and homologous tissue extracts from epidermis or spleen cells are capable of eliciting both transplantation immunity as revealed by the second-set reaction phenomenon and humoral immunity as revealed by the appearance of haemagglutinins.
Transplantation antigens and haemagglutininogens could thus be intimately related.

The activity of haemagglutinating sera seemed to be specifically inhibited by various preparations of transplantation antigens. Consequently a “haemagglutination inhibition test” has been developed as a quick and simple method for the study of transplantation antigens. So far, the biological assays (second-set phenomenon) and haemagglutination inhibition tests have always been in good agreement.

REFERENCES


DISCUSSION

Medawar: Brent and Ruszkiewicz, using “mucoid” type extracts (prepared in many cases by Prof. Albert’s and Dr. Lejeune’s methods) have also found that these extracts inhibit haemagglutination, they do somewhat prolong the life of homografts, but neither of these effects is in any degree specific. What one really wants for this work is something in mice equivalent to the ovarian cyst fluid in human beings, and I wondered if anyone working on transplantation antigens had systematically screened body fluids of mice to try to find out if any of them contained serologically active substances in a soluble form. The analysis of the human blood group substances would not have made anything like its present progress had it not been for Morgan’s fortunate discovery of the activity of ovarian cyst fluid.

Davies: There is such a substance in ascitic fluid. It seems to be a dominant agglutinogen of mouse cells, but this is a heat-stable substance, is species-specific, and has no connexion with H-2 antigen; this is Dr. Boyle’s “antigen 4”.

Hašek: Demonstration of such a sensitizing substance has been already published by V. Hašková and I. Hilgert (1961. Folia biol. (Prague), 7, 81). Using the skin-graft test these authors demonstrated sensitizing activity in the cell-free tumour ascitic fluid and in the cytoplasmic fraction of ascitic tumour cells.

Davies: Are these materials soluble or do they consist of membrane fragments?

Hašek: The originally tested material from cell-free ascitic fluid cannot be described as being in a soluble form, but it was prepared by centrifugation at 18,000–25,000 g.

Medawar: Do these materials contain lipid?

Hašek: Yes, they do.

Davies: The species-specific substance which my colleague Dr. Boyle has isolated does not contain lipid and is soluble. It is present on the surfaces of cells. We immunize rabbits with mouse cells, and this is the dominant agglutinogen. We feel that it is akin to blood group substance. I don’t think that it is a membrane fraction, in the sense usually used—that is to say, it is not insoluble.
**Brent:** To come back to the modest prolongation of survival of skin grafts produced by gastric phenol extracts: this is probably due to a general, non-specific suppression of the immune response of the animals. There are quite a few reports in the literature indicating that animals can be made very much more susceptible to bacterial infection by gastric extracts of this kind.

**Albert:** Dr. Lejeune suggested the same explanation of the prolonged survival of the skin homografts induced by his “mucoid extracts”; that is, a general depression of the immunological reactions.

As far as the soluble extracts are concerned, Dr. Lejeune and Dr. Castermans are studying comparatively the various fractions of their extracts from the point of view of the transplantation immunity induced in vivo and the haemagglutination inhibition in vitro. So far, any fraction which induces transplantation immunity also inhibits the haemagglutination reaction.

**Brent:** Does Dr. Lejeune have any idea why epidermal cells should be so very much more potent antigenically than lymphoid cells?

**Albert:** I don’t think he has any formal explanation; so far it has just been an observation. The difference of potency is even more impressive when epidermal and spleen cell extracts are used.

**Medawar:** Is it not possible that epidermal cells don’t contain the enzymes, certainly present in spleen cell extracts, which degrade these antigens? One of the difficulties with the type of preparation we use is that it is exceedingly unstable to incubation; but it is possible with these epidermal cell extracts, and also with ascitic fluid preparations of the kind that Dr. Davies has used, that these enzymes are simply not present, so that one doesn’t have this constant nuisance of a progressive loss of potency in the course of preparation. Has Dr. Lejeune tested the stability of his epidermal cell extracts to incubation? Does the haemagglutination inhibition potency of an epidermal cell extract decline very rapidly, as that of a splenic cell extract would do?

**Albert:** Dr. Lejeune tested the stability of his epidermal cell extracts at various temperatures: At 4° the potency of the extracts remains stable for 48 hours, after which it decreases rapidly. At 20° the potency declines very quickly and the extract loses nearly all its activity after 24 hours. At 37° the extract loses the major part of its activity after only one hour of incubation. The stability of the epidermal cell extracts
may, however, be preserved for several days when dried, without electrolytes, in the frozen state.

Woodruff: If you mixed the two, it would resolve the question of the enzyme destruction.

Davies: There does not appear to be any such enzymic degradation in the ascitic fluid, because this can be incubated overnight, and you don't find any drop in the haemagglutination inhibition titre, but this would not tell us whether antigenic potency had been affected.

Billingham: Since A and B blood group antigens are present on human epidermal cells (Coombs, R. R. A., Bedford, D. and Pouillard, L. M. [1956]. Lancet. 1, 461), the hair of mice and men might conceivably furnish a convenient source of blood group substances if means can be devised to extract and purify them.

Medawar: One must bear in mind that the form in which these antigens are present in ascitic fluids and in cell membranes is profoundly different: the one is probably glycolipid and the other certainly mucopolysaccharide; this may entail very great differences in methods of extraction.

Davies: Yes, but we do not know that this is true of H-2 or any other kind of antigen; it has only been proved for blood group substances.

Simonsen: Has anyone here tried to isolate the antigen from the liver? I recently heard rumours from America, where they claim that about 60 per cent of the total transplantation antigen that can be extracted from the mouse is concentrated in the liver.

Medawar: The Herzenbergs in America have made membrane fractions from liver which are certainly serologically active; the figure of 60 per cent is new to me.

Mitchison: May I return to the red cell again? The picture which seems to be emerging of the red cell here is one of a cell which has the antigens, but is non-immunogenic, and is therefore dismissed. I was wondering, on the other hand, about its tolerance-conferring properties. For instance, if a mouse tolerates a transfusion of incompatible red cells, is its response to a skin graft thereby altered? In my experience, mice don't eliminate incompatible red cells except in certain strain combinations. Pursuing the same line of thought: since this is transplantation antigen in some non-immunogenic form, what about the recombination experiment? Shouldn't red cells be mixed in with other tissue
DISCUSSION

cells, or with the antigens in an immunogenic form but with a different genetic specificity to see if they will then immunize? Something which I would like to interpret as a recombination experiment has been done in the case of the tuberculin reaction and the homograft reaction. Tuberculin injected alone doesn’t provoke cell-bound antibody, but if it is fed first to macrophages and these are then transferred to a foreign guinea pig that does provoke the formation of cell-bound antibody.

Barrett: I can’t let all this go without protesting. It seems that a great many people when studying the red cell find in their system results which lead them to the conclusion that the erythrocyte is not antigenic, in the sense of being able to provoke transplantation immunity in the general sense of that term. My laboratory does not yield this type of result: I find that, not only is the red cell a highly potent antigen for the generation of transplantation immunity, but so also are the carefully washed stromata of red cells; they are specific, they are highly antigenic, they are potent in relatively small doses.

Medawar: What strains are these, Dr. Barrett?

Barrett: I ordinarily have used DBA/2 as the donor and BALB/c as the recipient. I have also done it with C3H as the donor, and Dr. E. J. Breyere has done it with rats.

Davies: DBA/2 and BALB/c only differ at H-3 whereas C3H as donor would give H-2 differences also.

Barrett: Yes, but these results do not depend upon the compatibility at H-2. If the animal is compatible at H-2 one sees transplantation immunity but no haemagglutinins. If they are not compatible at H-2 then one may see both.

Hašek: Has anyone tried to estimate the presence of sex antigen on red cells? In our laboratory T. Hraba has done some experiments using \(^{51}\)Cr-labelled erythrocytes in C57BL mice, and he cannot find accelerated elimination of red cells of male and female in preimmunized animals, so it seems that this antigen is not present.

Silvers: We have attempted to induce tolerance of the Y factor with red cell preparations. Whereas as few as 200,000 leucocytes will consistently induce tolerance of the Y factor on inoculation into neonatal female recipients, the results with much higher dosages of erythrocyte preparations are extremely variable. As many as 100 million red cells
may sometimes give tolerance and we are convinced that this is due to contamination of our red cell preparations with leucocytes. We are, therefore, of the opinion that tolerance of skin cannot be induced with erythrocytes in mammals.

_Brent:_ Dr. Owen and I thought that the inability of red cells to sensitize might be because the red cell antigen requires adjuvant, and some years ago we investigated this possibility. We completely failed to get any sensitization against skin grafts with red cell-adjuvant emulsions. Dr. Barrett, have you repeated the experiments which you did originally with tumour tissues using skin grafts?

_Barrett:_ I have not, but Dr. Breyere has used some preparations in rats and tested them with skin grafts. Regrettably, his red cell preparations cannot be said to be certainly clear of leucocytes, although we can be sure that there aren’t a great many leucocytes because of multiple handlings in unsiliconized glass in the preparation of the antigen. However, the result he gets is compatible with the red cell dose and not compatible with the probable white cell dose.

_Billingham:_ Drs. D. Steinmuller and L. Weiner have recently found that injection of a suspension containing as few as 100,000 homologous B.N.-strain spleen cells into adult Lewis rats, weighing about 200 grams, will sensitize them in respect of B.N. test skin homografts transplanted 8 days later.

To return to the point raised by Dr. Mitchison: we have all tried, by various methods, to determine whether or not transplantation antigen specificity is present on red cells. One procedure that we are currently investigating takes advantage of the fact that the homograft sensitivity elicited by intraperitoneally inoculated splenic cells in mice dies away fairly rapidly. Inoculation of homologous red cells, or lyophilized tissues, should reawaken homograft sensitivity in these animals if they contain the appropriate antigenic configurations.

_Mitchison:_ I have no difficulty in procuring tolerance of skin grafts in chickens using red cells in irradiated blood; provided that the animal is tolerant of the red cells it seems usually to be tolerant of the skin graft. There are some technical complications about contamination of red cells by leucocytes, but I don’t think that the irradiated leucocytes were responsible for the tolerance. I would like to ask Dr. Hašek whether the compatibility of chromium-labelled red cells from the male in the
female is a valid criterion of presence of antigen? If you take a combination where you know there is an antigenic difference, do you then get accelerated clearance of the foreign red cells?

*Hassek:* We have not tested this question systematically.

*Amos:* Sachs and Heller published some negative results in the mouse of the Y antigen on the red cells; the antigen was present in spleen and liver.

To go back again to the question of enzyme inactivation: I wonder whether the efficiency of the irradiated cells that Revesz used was partly due to loss of enzymic activity in the cells. We have compared the efficiency of 20 million X-irradiated *versus* smaller doses of living, and also a formol-acetone extract and a citric acid extract preparation of tumour cells to immunize against a challenge. We found that while the X-irradiated were more efficient than the live cells, both the formol extract and the citric acid extract, and especially the citric acid, gave us a very great degree of promotion.

*Russell:* We have tried to induce tolerance with *in vitro* irradiated splenic cells in the hope that we could produce tolerance without runt disease (this would be the sort of thing that you were talking about). So far we haven’t been able to find any dosage which will eliminate the production of runt disease without also eliminating antigenic activity.

*Hildemann:* I wonder if Dr. Mitchison’s success in producing tolerance in chickens with red cells might not be attributable to the fact that these are nucleated red cells. In our own experience testing the antigenic potency of the Syrian hamster red cells versus buffy-coat cells, the degree of skin homograft immunity induced was proportional to the number of blood leucocytes present in the inoculum. As Billingham has pointed out, many of us have observed that a very small dose of leucocytes is antigenically effective. I know of no method for obtaining absolutely pure preparations of red cells to make an unequivocal test.

*Davies:* One can make a good separation of red cells and white cells by centrifuging through a sucrose gradient.

*Brent:* I think there may possibly be a way round the difficulty in this running battle of Barrett *versus* the rest. In your experiments, Dr. Barrett, you are using two strains in which there is some measure of incompatibility—if I remember rightly, about 10 per cent of the animals
respond to the tumour and reject it, and the other 90 per cent don’t. This suggests, in the first place, that even in animals which do not throw off their tumours, there is a low level immune response which the tumour is able to override by some means or other. Now, if by the injection of red cells, or of red cell stromata, you are eliciting the formation of haemagglutinins and other serum antibodies, could it not be that in this precariously poised system you are producing a syner- gistic effect of the type described by Gorer and Batchelor, so that the presence of the serum antibodies is emphasizing the natural response against the tumour and is just tilting the balance?

Barrett: In the first place, I don’t want to be misunderstood as having said that all antigens are present on the red cell, nor on the other hand, that all red cells of all strains must necessarily give the response that I have got; all I am saying is that in my system it is there. Secondly, I don’t want to be misunderstood, as I have been sometimes, as saying that the white cell is not antigenic in this regard; of course it is antigenic and I suspect that it is more strongly antigenic than the red cell, although I have never put this to the test in my system. Thirdly, in my system, as to the contamination of white cells, I was very careful not to make any strong statement about that, but some of you will recall that in this system, when I take all the white cells that can be removed, from ten times the dose that I use for my immunization, these cells only produce half as much effect as do my red cell preparations in which I can find no white cells whatever—though I don’t deny that some might get by. Fourthly, as regards the last point that Dr. Brent made: 10 per cent of these animals are on the average resistant to this tumour; however, there are no haemagglutinins generated by any of these procedures, either by multiple inoculations of the tumour, or by ten or fifteen times as much dose of the blood inoculum.

Nakić: I would like to go back to something Dr. Russell said about irradiating spleen cells and trying to induce tolerance in vitro. Do you think that irradiation might reduce the antigenic power of the spleen cells?

Russell: I think it must clearly do so if the dosage is sufficiently high to obviate self-replication of the cells of the inoculum. Perhaps what I should do in further experiments is to give repeated doses of irradiated cells because I may have missed tolerance, since it died away before I
DISCUSSION

tested for it. I am fully aware of this. All I say is that I haven’t found it.

Nakic: I remember some experiments performed by Howard and Michie who attempted to induce tolerance by repeated injections of irradiated spleen cells followed by normal non-irradiated spleen cells and, as far as I know, they obtained no tolerance in newborn animals. That is a point in which I am very much interested.

Michie: We have subsequently tested the immunizing power of these irradiated preparations, and, although our estimates are still rather vague, it looks as though there is a weakening of immunizing power by a factor of more than ten and less than a thousand.

Silvers: Was this in C57-CBA?

Michie: We used two combinations in studying tolerance: A with CBA, and CBA with C57.

Silvers: Since CBA and C57 is a very difficult combination, I wonder if a similar experiment with CBA and C3H might be worth while.

Russell: I think a point that Billingham has made in this regard is that if you inject a living, self-replicating inoculum composed of cells which can respond to the foreign antigens that surround them, then you have an increasing dose of antigen since antigenic stimulation will lead to cell multiplication, which may have some bearing on this.

Michie: That could fully explain it.

Hildemann: It seems that there is a controversial point in the findings of Lejeune-Ledant and Medawar. If I understood Lejeune-Ledant’s presentation correctly, he was able to get immunity with soluble extracts by the intravenous route, and was also able to obtain transplantation immunity without the production of haemagglutinins. However, when large amounts of any of these antigenic preparations were injected serum antibodies were also detected. Perhaps this apparent disparity between the results of Lejeune-Ledant and Medawar is attributable to subtle differences in the antigenicity or chemical composition of the preparations injected. I wonder, though, whether too high a degree of specificity is attributed to the haemagglutinins relative to the antigenic character of the soluble extracts. In the earlier work that Prof. Medawar and I did together—admittedly with crude preparations—we were able to get transplantation immunity in the absence of detectable serum antibodies. We wondered at that time if other methods might
reveal isoantibodies which were not apparent in dextran-haemagglutination or haemolytic tests. But if, as Prof. Medawar's work clearly indicates, the antigens show chemical heterogeneity, there is all the more reason to believe that the serum antibodies induced will also be heterogeneous in their specificities. I think we may give too much weight to the red cell antibodies and their titres under these circumstances.
H-2 HISTOCOMPATIBILITY ANTIGENS OF THE MOUSE

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The purification of antigens which distinguish one kind of inbred mouse strain from another may be followed by a variety of methods, including, for example, the hastening of allogeneic skin graft rejection, the enhancement of tumour growth in allogeneic strains, and inhibition of haemagglutination. There are other possibilities which have not been taken advantage of for this purpose (Simonsen, 1961). The skin grafting and enhancement tests have an unfavourable dose-response relationship but in spite of this they have been used with considerable success.

Although the general feeling is that the specificity of transplantation (T), enhancing (E) and histocompatibility (H) antigens is likely to be basically the same, there are differences which have to be explained. The answer to such problems should be within reach when purified products are available for study. The work described here is a contribution to our knowledge of H-2 antigens, a haemagglutination inhibition test having been used to follow purification. The test limits one to antigens whose distribution is controlled by the H-2 locus, since other H antigens are not usually detectable on red cells by this method. The relationship of the products to T and E antigens will be described in due course.

For T, E and H antigens we have the information that activity is closely bound to the insoluble structural part of the cell (Billingham, Brent and Medawar, 1956, 1958; Kandutsch and Reinert-Wenck, 1957; Kandutsch, 1960; Herzenberg and
Herzenberg, 1961), that DNA and RNA are not components of the antigens (Hašková and Hrubešová, 1958; Castermans, 1961, and others), and that protein, and also probably carbohydrate and lipid are present in the preparations of the T, E and H antigens so far obtained (Kandutsch, 1961). The most advanced preparations would seem to be those of Medawar (T), Kandutsch (E) and Herzenberg (H), but it has not been established that the least entity able to carry activity has yet been obtained in any instance.

**Experimental procedures**

**Animals and tumours**

Mice of strains A, C3H/He, C57BL and BALB/c have been used; each of these was mated at random within its colony for up to three generations from a strictly inbred breeding nucleus. Ascites tumours specific for these strains were Sa-1, BP8, EL4 and CL2 respectively; these tumours are well known and adequately described in the literature. The Landschütz subline of the Ehrlich ascites carcinoma (abbreviated LAN) has been used as a “non-specific” cell for comparative purposes.

H-2 antisera have been prepared in mice by three or four weekly subcutaneous injections of appropriate tumour cells or spleen cells. Immune ascitic fluid, induced with LAN injected intraperitoneally five days before the last immunizing injection, has generally been used as source of antibody since this gives a better yield than mouse serum. To avoid confusion, however, this will be referred to hereinafter as antiserum.

Rabbit anti-mouse sera were prepared by immunization with ascites tumour cells, or with tumour cell ghosts prepared by treatment with strong salt solution until no more soluble material could be extracted (Haughton and Davies, 1962). A preliminary immunization using Freund’s adjuvant was followed by repeated intravenous injections until an adequate response, determined by agar diffusion, had been achieved.
Serological methods

In order to follow the progress of purification, the dextran-normal human serum (NHS) method of inducing haemagglutination with H-2 antisera (Gorer and Mikulska, 1954) has been used for inhibition tests. These have been described by Davies and Hutchison (1961), but are briefly as follows. For whole cells, or for insoluble but dispersible preparations, an equal volume of suspension was added to all tubes of a series of antiserum dilutions (prepared in 1 per cent dextran) and incubated. After centrifuging, samples of the supernatant were reincubated with red cells (in 1 : 2 NHS which had been inactivated and absorbed with similar red cells) and haemagglutination read microscopically. The difference between the haemagglutination titre thus obtained and the titre of the unabsorbed antiserum was taken as the measure of activity.

For soluble preparations and those giving stable suspensions or emulsions, antiserum was used in a small number of haemagglutinating doses and the potential inhibitor diluted out to give an inhibition titre. Dextran and NHS were also included in this system.

For following purification it is considered essential to use a monospecific antiserum, because if different H-2 specificities are carried by different chemical entities a separation of these on fractionation would be obscured by a polyspecific antiserum. Although it is possible that the H-2 "antigens" really represent different structural features of one molecular complex, as for example in the "O" somatic antigens of the Enterobacteria (Davies, 1960), in a system based on pseudo-alleles this is less likely to be the case. Some of the antisera obtainable using the four mouse strains available are shown in Table I; relatively monospecific sera for H-2-C, D, D^b, D^k, E, E^d, F and K are thus available without resorting to absorption methods.

Agar diffusion was carried out as described by Crumpton and Davies (1956).
Table I

PRODUCTION OF H-2 ANTISERA

<table>
<thead>
<tr>
<th>Mouse strain immunized</th>
<th>Injected cells</th>
<th>H-2 specificity using red cells from</th>
<th>A</th>
<th>C3H</th>
<th>C57BL</th>
<th>BALB/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C3H</td>
<td>—</td>
<td>D^k</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>C57</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>D^b</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>BALB/c</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>E^d</td>
</tr>
<tr>
<td>C3H</td>
<td>C57</td>
<td>F</td>
<td>—</td>
<td>—</td>
<td>D^bF</td>
<td>F</td>
</tr>
<tr>
<td>C3H</td>
<td>BALB/c</td>
<td>DF</td>
<td>—</td>
<td>F</td>
<td>—</td>
<td>DE^dF</td>
</tr>
<tr>
<td>C57</td>
<td>C3H</td>
<td>CK</td>
<td>—</td>
<td>—</td>
<td>C</td>
<td>—</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C3H</td>
<td>EK</td>
<td>D^kEK</td>
<td>E</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(C3H × C57)F_1</td>
<td>A</td>
<td>D</td>
<td>—</td>
<td>—</td>
<td>D</td>
<td>—</td>
</tr>
<tr>
<td>(C3H × BALB)F_1</td>
<td>C57</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>D^b</td>
<td>—</td>
</tr>
<tr>
<td>(C57 × BALB)F_1</td>
<td>A</td>
<td>K</td>
<td>K</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Chemical methods

The chemical methods used have been standard procedures: nitrogen by Kjeldahl; phosphorus by Fiske and Subbarow; total protein by ninhydrin after acid hydrolysis; total carbohydrate by orcinol; nucleic acid by absorption at 260 mμ; hexosamine as described by Rondle and Morgan (1955) and sialic acid by the method of Warren (1959).

Purification of H-2 antigen

Preliminary experiments

When tumour cells were tested for their ability to absorb haemagglutinating antibody from H-2 antisera, it became clear that they carried H-2 specificity on their surfaces. For these experiments blood cells were removed from the tumour-cell suspensions by sedimenting through a sucrose gradient. The presence of H-2 specificity on the tumour cell surface could be shown more clearly by mixed agglutination (Coombs, Bedford and Rouillard, 1956) when dextran and NHS were used in the system and non-specific cells (LAN) as control (Boyle, unpublished). The examination of ascitic fluids from which the
cells had been removed showed that specific activity was present there also, apparently in soluble form. Although the level of activity was low (inhibition of haemagglutination at 5–10 mg./ml.) this seemed a favourable starting material for isolation of active material since ascitic fluid is similar to serum, and of simpler composition than cell homogenates.

Ascitic fluid recovered after growth of tumour for six to ten days (BP8/C3H) had a lower level of activity than that recovered after 13 days. The level seemed to decrease again from 14 to 16 days. Preliminary experiments indicated that fractionation of ascitic fluid with ammonium sulphate gave little concentration of activity in any fraction but that active material adsorbed non-specifically on to precipitates formed at various levels of salt concentration. Attempts to elute activity from diethylaminoethyl (DEAE)-cellulose, carboxymethyl (CM)-cellulose, or starch (for block electrophoresis) within reasonable limits of pH were unsuccessful, and eluates of freeze-dried ascitic fluid contained very little active material.

The capacity for non-specific adsorption indicated the necessity of finding some way of obtaining a substantial degree of purification in the initial step if severe losses of active material were to be avoided. A possibility of achieving this arose from the observation that activity could be recovered in the fraction precipitated from ascitic fluid by simple dialysis against distilled water. This also showed that activity was associated with some particular molecular fraction and led to the feeling that the behaviour in previous experiments might be due to lipid being associated with the active substance.

**Large-scale extraction**

Mice were used in batches of 1,000; LAN and BP8/C3H peritoneal contents were recovered directly by suction into aspirators, citrate being used as anticoagulant. During the growth of EL4/C57 and CL2/BALB/c little free fluid was
produced and it was necessary to wash out the cells, which was done with pH 7·2 buffered saline (BS). Some relevant data are shown in Table II. The LAN "non-specific" tumour fluids (this tumour is not altogether without specificity—see below) on centrifuging give packed cells occupying half of the total volume of the fluid and therefore show an artificially low value for the weight of soluble material in the ascitic fluid after dialysis and freeze drying. The two leukaemias, CL2 and EL4, give a very small yield when compared with LAN, BP8 and other tumours tested, but give a relatively higher yield of H-2 antigen.

Table II

Mouse tumour products

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Mouse strain</th>
<th>Days of growth</th>
<th>Vol. ascitic fluid (l.)</th>
<th>Wt. cells (g.)</th>
<th>Wt. soluble fraction (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAN</td>
<td>A</td>
<td>15</td>
<td>12</td>
<td>200</td>
<td>200</td>
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<tr>
<td>LAN</td>
<td>outbred</td>
<td>15</td>
<td>14</td>
<td>200–300</td>
<td>200–250</td>
</tr>
<tr>
<td>BP8</td>
<td>C3H</td>
<td>13</td>
<td>6–8</td>
<td>140–200</td>
<td>115–120</td>
</tr>
<tr>
<td>CL2</td>
<td>BALB/c</td>
<td>11</td>
<td>4</td>
<td>50–60</td>
<td>80–110</td>
</tr>
<tr>
<td>EL4</td>
<td>C57</td>
<td>11</td>
<td>4</td>
<td>8–12</td>
<td>16–25</td>
</tr>
</tbody>
</table>

Peritoneal contents were centrifuged slowly at room temperature to separate tumour cells from red cells; LAN was chosen in preference to the Ehrlich, Krebs, and other non-specific tumours tested, because it was less haemorrhagic. EL4 generally gave few red cells in the peritoneum but BP8 and CL2 usually required repeated slow sedimentation from BS before the red cells were wholly removed. The washed tumour cells were freeze dried and stored at −20°C to await extraction of antigens other than H-2. The BS washings of tumour cells had little H-2 activity and were discarded. The original ascitic fluid supernatants were centrifuged at high speed in a continuous flow centrifuge (Alfa Laval) to remove residual cells and debris before dialysis.

Dialysis against distilled water gave an insoluble fraction of
greatly enhanced activity and activity could not be detected in the soluble fraction. By fractional precipitation of fluid with water, or by fractional dialysis of fluid against buffers of low ionic strength, it was found that the dialysis precipitate (DP) fractions contained inactive material. The resolving power of the method was poor, however, and whereas material giving haemagglutination inhibition at 50 μg./ml. could be obtained, there was considerable loss of activity in adjacent fractions. The following procedure was therefore adopted: fluid was diluted with 0.75 vol. of distilled water and left at 2–3° overnight. The insoluble material which appeared was almost inactive and was removed by being centrifuged, resuspended in water, dialysed and freeze dried. The supernatant solution was dialysed against slowly running distilled water for 40 hours at 2–3° and the insoluble fraction, which carried most of the activity, was recovered by being centrifuged in the cold. This fraction gave inhibition at about 100 μg./ml. and was obtained in the following yields from successive batches of BP8/C3H: 0.95 g., 2.0 g., 1.68 g., 1.33 g. The total weight of precipitable material obtainable from original cell-free ascitic fluid is 3.0 g. (approximately 2.5–3.5 per cent of the total dry weight), so that rather more than half of this is usually obtained in the active DP fraction and the remainder appears in the inactive 0.75 vol. water dilution precipitate. The variation in the weights of products is due to differences in the average weight of mice in the batches at the time of tumour inoculation. Yields from CL2 and EL4 are, of course, smaller.

When normal mouse serum is dialysed under similar conditions the insoluble fractions amount to about 12 per cent of the total material but no activity is present.

The active DP fractions give suspensions in BS which are moderately stable. Fractional centrifugation showed that all activity was in a fraction which could be sedimented at 80,000 g for two hours, leaving 80 per cent of the weight of material in the supernatant fluid, and although activity at the level of
5 μg./ml. could be obtained in fractions sedimented at intermediate speeds, much activity was left in adjacent fractions. In any case, as the active sediments gave only moderately stable suspensions in BS, their differing particle sizes would be likely to affect their measurable activity. Fractional centrifuging was therefore subsequently omitted and advantage taken of the sedimentation to remove all soluble components by centrifuging four times from BS, twice from 0.9 per cent salt, and then twice from water. Tests by agar diffusion now showed no significant

Table III

Analysis of ascitic fluid and its products

<table>
<thead>
<tr>
<th>Material</th>
<th>Wt. (g.) from 1,000 mice</th>
<th>N (%)</th>
<th>P (%)</th>
<th>Lipid (%)</th>
<th>CHO (%)</th>
<th>Activity (†)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascitic fluid</td>
<td>120</td>
<td>13.3</td>
<td>0.3</td>
<td>6.0</td>
<td>2.5</td>
<td>1–2</td>
</tr>
<tr>
<td>³/₄ vol. H₂O precipitate</td>
<td>1.5</td>
<td>13.0</td>
<td>0.7</td>
<td>12.5</td>
<td>5.8</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Dialysis precipitate</td>
<td>1.5</td>
<td>13.4</td>
<td>0.7</td>
<td>10.5</td>
<td>2.5</td>
<td>100–200</td>
</tr>
<tr>
<td>Dialysis supernatant</td>
<td>115</td>
<td>14.1</td>
<td>0.2</td>
<td>5.6</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Spinco precipitate</td>
<td>0.3</td>
<td>10.6</td>
<td>1.2</td>
<td>30.4</td>
<td>4.0</td>
<td>500–1000</td>
</tr>
<tr>
<td>Spinco supernatant</td>
<td>1.2</td>
<td>14.1</td>
<td>0.4</td>
<td>4.0</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>TT‡</td>
<td>0.04</td>
<td>9.8</td>
<td>1.2</td>
<td>26.4</td>
<td>3.0</td>
<td>0</td>
</tr>
<tr>
<td>TM1</td>
<td>0.10</td>
<td>8.4</td>
<td>1.1</td>
<td>40.3</td>
<td>3.5</td>
<td>0</td>
</tr>
<tr>
<td>TM2</td>
<td>0.13</td>
<td>10.4</td>
<td>1.0</td>
<td>35.35</td>
<td>3.7</td>
<td>200–400</td>
</tr>
<tr>
<td>TB</td>
<td>0.03</td>
<td>9.0</td>
<td>1.2</td>
<td>25.2</td>
<td>9.5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Carbohydrate.
† Dilution giving haemagglutination inhibition divided by 100.
‡ Tartrate gradient fractions.

reaction for components of normal serum. The activity at this stage was generally 10–20 μg./ml., and analytical figures are shown in Table III.

Advantage having been taken of solubility and of mass, the increase in the proportion of lipid with increasing activity suggested the use of density gradients. Potassium tartrate was chosen on account of its low viscosity (McCrea, Epstein and Barry, 1961). The centrifuged precipitate (~300 mg.) was suspended in water at 2 per cent concentration, centrifuged for 20 minutes at 1,000 g to remove dirt, and 1 ml. volumes layered
on to gradients running from 10 per cent to 40 per cent tartrate (density 1.06 to 1.23). After centrifuging at 95,000 g to equilibrium, a number of bands appeared which were recovered separately, and dialysed free from salt. Samples of solution were tested for activity and the remainder freeze dried for analysis. All but two bands were inactive and subsequently the fractions were recovered as follows: top fraction (TT), upper middle band (TM1), lower middle band (TM2) and bottom fraction (TB). The middle fractions together account for 60–80 per cent of the SP (Spinco precipitate) fraction. Typical analyses are shown in Table III, where most figures are averages from at least ten experiments.

Both the TM1 and TM2 fractions had the property of inhibiting in the haemagglutination test, but only TM2 fractions induced haemagglutinating antisera when injected in appropriate allogeneic mouse strains.

When TM2 fractions are recycled through new tartrate gradients material is again obtained in the TT and TB regions. This suggests that the active material is not stable in strong salt, and although the data given below are for tartrate gradient materials, sucrose gradients are now being used over the same density range for increased stability. For these gradients activity is found in a central band (SM) and the material recovered, after dialysis to remove sucrose, gives the same analytical figures as TM2 fractions (Davies, 1962).

Hazards of contamination

It is not practicable to recover the products from batches of 1,000 mice under sterile conditions and from time to time organisms have appeared which grow readily at low temperature and are resistant to antibiotics. Various means of sterilization tested have proved harmful to the H-2 antigen, except u.v. irradiation. After the first sedimentation in the Spinco, the resuspended precipitate is therefore distributed in dishes to a
depth of not more than $0.5$ cm. and exposed to u.v. lamps for 15 minutes.

**Properties of the product**

**Chemical and physical properties**

The final antigenic fraction (TM2 or SM) gives a stable milky suspension in water but is precipitated by the addition of salt (e.g. $0.1M$–NaCl). The antigen is insoluble in dimethylsulphoxide, formamide, dimethylformamide, dioxane, sodium thioglycollate solution, or mixtures of chloroform–methanol–water, and is only partly soluble in $6M$–urea or guanidine–HCl. It becomes more stable and less opalescent in $0.2$ per cent sodium dodecyl sulphate, or $66$ per cent acetic acid, or $0.2$ per cent deoxycholate at pH 9 (tris-phosphate buffer), or $1$ per cent lysolecithin (but not sphingomyelin), or in $50$ per cent aqueous chloro-ethanol containing $0.5M$–sucrose. If the pH is raised from 9 to 11, material of a higher nitrogen content is precipitated, leaving a low nitrogen (3 per cent) component in solution. No solvent has been found in which serological activity is retained, or which gives retention of activity when the solvent is replaced with water by dialysis.

Activity is labile to acid (pH 5), to alkali (over pH 9.0) and to heat ($60^\circ$, 20 min.); treatment with ultrasonics (M.S.E.—Mullard drill) disperses the material to an opalescent, more stable solution, but there is loss of activity (Fig. 1). Little activity can be found after freeze drying, or even after freezing and thawing a suspension, so purification can only be carried out starting with fresh ascitic fluid, and it has to be carried to completion without freeze drying at any stage. Extraction with organic solvents, or exposure to $1$ per cent Tween–20 (polyoxyethylene sorbitan monolaurate), results in loss of activity.

The chemical composition of the products is shown in Table III. The active substance is a lipoprotein, containing $0.5$ per cent sulphur and not over $1$ per cent hexosamine. There is $0.3$ per
cent sialic acid, but no uronic acid, although the material is markedly acidic. The products from successive batches of mice vary considerably in their proportions of lipid and protein and, taking the nitrogen content of the "lipid-free" residue as 14·5 per cent, the proportions of lipid and protein can be found from the percentage of nitrogen in the TM2 fraction. The nitrogen content of the products of different batches has varied from 11·0 to 8·5 per cent, with lipid from 30 to 50 per cent. The nitrogen content of the TM1 fractions is slightly lower than that of the TM2.

The lipid and protein components can be largely separated by solvent extraction. Removal of lipid with ether at $-15^\circ$, using poorly active freeze-dried material, resulted in little loss of

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Fig. 1. The effect of ultrasonic vibration on the haemagglutination inhibiting activity of H-2 antigen.
activity in the residue but only about one-third of the total lipid is removed in ether. Extraction in the same way with chloroform/methanol (2 : 1) takes out two-thirds of the lipid and leaves an inactive residue. Refluxing with ether until no more lipid is extracted gives nearly half the total lipid and most of the remainder can be removed by refluxing repeatedly with chloroform/methanol. The relatively low nitrogen content of the residue suggested that lipid might not have been totally removed by this treatment; when the material was hydrolysed with strong HCl at 100° overnight, fatty acids could be extracted with ether and they accounted for 10 per cent of the weight of the residue in terms of phospholipid. There is, therefore, some closely bound lipid which cannot be removed by solvent extraction and this is not included in the lipid figures given in Table III. The main ether-soluble lipid fraction has 0.8 per cent nitrogen and 1.6 per cent phosphorus but no reducing sugars. The chloroform/methanol-soluble fraction contains 2.5 per cent nitrogen, 2.9 per cent phosphorus and 5 per cent carbohydrate. On hydrolysis of this fraction with N-H₂SO₄ for eight hours, followed by neutralization with Ba(OH)₂ and removal of charged hydrolysis products by passing through ion-exchange columns, chromatograms revealed the presence of glucose and galactose. The proportions of these sugars have differed in the products of different mouse strains but it is not yet clear how reproducible these differences are. Hydrolysis for only 20 minutes and subsequent treatment in the same way reveals a third sugar on chromatograms which has not been identified. Attempts to test the lipid fractions for serological activity by suspension as emulsions have not been successful although SP active fractions show full activity when emulsified in cholesterol and lecithin.

The protein remaining after solvent extraction by refluxing is very insoluble. It has 14.5 per cent nitrogen, 0.6 per cent phosphorus and less than 2 per cent carbohydrate and after strong hydrolysis chromatograms show at least 15 amino acids (Asp,
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Glu, Pro, Ser, Thr, Gly, Ala, Val, Leu, Tyr, Phe, CysSH, Lys, Arg, and one unidentified).

Homogeneity

Evidence for homogeneity proves very difficult to obtain. The products are essentially free from all ascitic fluid components which are soluble in saline or water and have only one serological specificity (mouse species specificity) other than that of histocompatibility (see below). They behave as single components by density. Further evidence is limited to experiments in which impurities cannot be found under circumstances where little information can be obtained about the behaviour of the bulk of the material. Analytical ultracentrifugation of SP fractions in water shows a rapid clearing of opalescence between 20,000 and 30,000 rev./min. (Spinco Model E) followed by a single peak representing about 10 per cent of the material, which sediments with rapid diffusion. The TM2 and SM fractions examined in the same way show a similar rapid clearing of opalescence to give a gel on the bottom of the cell, but there is no indication of a second component. At pH 9.0 (tris-phosphate buffer) a similar picture is found, showing that the second component in the SP fraction is not a degradation product of the H-2 antigen. Electrophoresis on cellulose-acetate paper at pH 9 gives no movement of protein or lipid-staining material from the starting point, showing that at this pH no material was present which might have failed to separate from the meniscus in the ultracentrifuge run at the same pH, and that at pH 7 to 9 the material behaves as a single substance.

Ultracentrifuge runs have also been carried out under conditions where degradation might be expected to have occurred. Solutions in 0.2 per cent sodium dodecyl sulphate at pH 8 showed a single sedimenting component but at pH 6.5 there was a separation of two peaks having proportions of about 4:1. In deoxycholate at pH 9 some material is present which fails to break away from the meniscus. Runs in solvents which give relatively clear
solutions have either been too dense (e.g. 6M-urea), or given micelle formation (lysolecithin). There is therefore no evidence for inhomogeneity, but only suggestive evidence for physical homogeneity. The latter is, however, enhanced by the finding that the material sedimented in the ultracentrifuge, when resedimented in three fractions at successively increasing $g$ values, cannot be distinguished by chemical analysis.

**Serological properties**

Information on immunological homogeneity has been obtained by agar diffusion, using rabbit anti-mouse sera. Mouse anti-mouse-H-2 sera do not give precipitin lines with H-2 antigen preparations in agar presumably because H-2 antibody is "incomplete". Since ascitic fluid has a composition similar to that of serum, though with a smaller total concentration of solutes (35 mg./ml. after dialysis), the extent of removal of serum components can be checked. Globulins compose the greater part of the 0.75 vol. water dilution precipitate, but in Fig. 2a it will be seen that they are also present in large amount in the DP fraction, although some other components have been substantially removed. The Spinco supernatants contain the bulk of the remaining serum components; four sedimentations leave an SP fraction in which two serum components are still detectable, one of these being globulin, but six sedimentations reduce these to a negligible level. The final TM fractions are free from serum components as tested at a level to show contamination down to 1 per cent.

Rabbit anti-mouse-cell sera, after absorption with normal mouse serum, show six main lines due to cell-bound antigens (Haughton, Boyle and Davies, 1962). No single serum is available which possesses all six antibodies in detectable amounts. In Fig. 2b a serum having several of these components is shown reacting with the H-2 products and it can be seen that the final TM2 fraction contains one of these antigens only, but this at a high level of activity. This antigen (antigen no. 3, Haughton, 1962)
Fig. 2. Agar diffusion line patterns of ascitic fluid, H-2 antigen, and intermediate fractions tested against

(a) rabbit antiserum against normal mouse serum (in central wells);
(b) rabbit antiserum against mouse cells, after absorption with normal mouse serum (in central wells).

Peripheral wells all contain 330 μg. of antigen, labelled thus:

A = Ascitic fluid.
B = Dialysis precipitate.
C = Spinco precipitate.
D = TM2 gradient fraction.
E = $\frac{3}{4}$ volume water dilution precipitate.
F = Dialysis supernatant.
G = Spinco supernatant.
H = Pooled top plus bottom gradient fractions.

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is a structural component of mouse cells, believed to be associated with cell membranes, but it can be found in soluble form where rapid growth is in progress, e.g. serum of pregnant mice, and ascitic fluid. It is a protein and has species specificity but no special specificity for different mouse strains. The activity of the TM2 fraction with respect to this specificity indicates that it must be the protein component of the H-2 lipoprotein.

Table IV

Inhibition of haemagglutination by H-2 antigens in polyspecific H-2 antisera

<table>
<thead>
<tr>
<th>Antigen from C3H (H-2-CDkEfK)</th>
<th>Serum 1</th>
<th>Serum 2</th>
<th>Serum 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,000</td>
<td>2,000</td>
<td>1,000</td>
<td>500</td>
</tr>
<tr>
<td>* Serum 1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>† Serum 2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>‡ Serum 3</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

Antigen from C57 (H-2-cD^bEfK)

| Serum 1 | — | — | — | — | — | — | — | — | — | — | — | 1 | 3 | 4 |
| Serum 2 | — | — | — | — | — | — | — | — | — | — | — | — | 1 | 3 | 4 |
| Serum 3 | — | — | 2 | 2 | 3 | 3 | 4 | 4 | 3 | 4 | 4 | 4 | 4 |

Antigen from BALB/c (H-2-CDE^dFk)

| Serum 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Serum 2 | — | — | 3 | 3 | 3 | 4 | 4 | 4 | 3 | 4 | 3 | 4 | 4 | 4 | 4 |
| Serum 3 | — | — | 3 | 3 | 3 | 4 | 4 | 4 | 3 | 4 | 3 | 4 | 3 | 4 |

* BALB/c anti-C3H serum at 1:150, tested with C3H red cells, (detect D^kEfK)
† BALB/c anti-C57 serum at 1:150, tested with C57 red cells, (detect D^bE)
‡ C3H anti-BALB/c serum at 1:150, tested with BALB red cells, (detect D^dF)

Degrees of agglutination from 4 = maximum to 1 = minimum.

The specificity of the products of the different mouse strains has been tested using some of the sera shown in Table I. The homologous and heterologous reactions with three polyspecific H-2 antisera are shown in Table IV, from which it is clear that strong inhibition of haemagglutination occurs when the inhibitor is prepared from the mouse strain used for immunization and weak inhibition where cross-reactions might be anticipated. These highly active inhibitors are quite inactive in systems where no cross-reactions are to be expected.
Tests with some monospecific H-2 antisera are shown in Table V; qualitative inhibition entries are given in this table but again the results clearly indicate that the H-2 inhibitors carry the specificities expected from the known distribution of H-2 alleles.

Table V

HAEMAGGLUTINATION INHIBITION BY H-2 ANTIGENS IN MONOSPECIFIC H-2 ANTISERA

<table>
<thead>
<tr>
<th>Antigen from</th>
<th>Serum specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-2-C</td>
</tr>
<tr>
<td>C3H</td>
<td>+</td>
</tr>
<tr>
<td>C57BL</td>
<td>-</td>
</tr>
<tr>
<td>BALB/c</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = substantial inhibition
- = no inhibition at 5 mg./ml.

In the course of extraction and purification, where C3H mice were mainly used with an H-2–K serum to follow activity, products of LAN grown in C3H or outbred mice were used to show lack of activity in the products of this “non-specific” tumour. An unexpected finding with the TM2 fraction of this tumour ascitic fluid has been that it is not in fact devoid of H-2 specificity, but is able to inhibit both H-2-C and H-2-F haemagglutination.

TM1 fractions, when used to immunize mice from different strains, have not yet induced an antibody response. TM2 fractions are antigenic and, injected into appropriate mouse strains, induce antibodies whose haemagglutinating activity can be inhibited only by material having the H-2 specificities of the injected material.

Discussion

Homogenates of whole cells are extremely complex mixtures of substances, from which the isolation of homogeneous components is a most difficult task. This is particularly true if the
desired product is present in small amount, when its chemical nature is not known and when tests for activity leave much to be desired. This situation holds for T, E and H antigens except that we cannot be sure that they are present in small amount: indeed there are indications to the contrary. The components of serum and hence of ascitic fluid have been extensively studied and although ascitic fluid is more complex in that it contains tumour cell products, isolation from this source seemed to offer more favourable prospects. With a knowledge of the chemical nature and properties of an H-2 antigen, isolation from cells should become a much less difficult task. Incubation of washed tumour cell suspensions at $37^\circ$ for 24 hours under toluene gives an extract from which active H-2 inhibitors can be concentrated by the dialysis steps described, but characterization of the ascitic fluid product is still in progress and is to be completed before examination of the product of cell extraction.

Information on homogeneity of the essentially insoluble product is singularly difficult to obtain, but no evidence for the presence of impurities has yet been found. It is not suggested, however, that the least chemical entity able to carry H-2 specificity has been obtained and it may yet be possible to remove parts of the complex without impairing biological properties. Unfortunately it has not been possible to obtain a true solution with full activity, which would allow one to fractionate by the high-resolution methods now available.

The product, as it stands, is lipoprotein. The presence of a few per cent of sugars leaves the possibility that activity is associated with an independent carbohydrate present in small amount. This seems very unlikely, however, in view of the inhibition activity at high dilution (in spite of poor dispersity) and the fact that most of the carbohydrate is present in the lipid fraction as glycolipid.

The protein component has been identified as an antigen in its own right and probably dissociates from the H-2 antigen. It has a
specificity of its own quite distinct from H-2 specificity. The activity of this protein component, as measured by dilution in agar diffusion, is virtually as strong as that of the “antigen 3” isolated from non-specific tumour fluids, or cells, where it is believed to be associated with the membraneous structures and composes a part of the cell which persists in insoluble form after exhaustive extraction with salt solutions (from 1 to 15 per cent) and with water (Haughton and Davies, 1962). Attempts to find H-2 specificity in this protein after removal of lipid from the H-2 antigen have not been successful, though the methods used for lipid removal so far are very unsatisfactory.

The lipid component has not been properly examined but there is every indication that it is a complex of different lipids. The proportion of lipid in successive extractions has varied considerably and it seems possible that dissociation may occur in the course of preparation. It is not yet clear how closely bound the protein and lipid moieties really are.

Information about the immunological determinant parts of the complex has not yet been obtained, but many facts point to the lipid component being involved. The proportion of lipid increases with increasing activity on purification, and the protein component has non-H-2 species specificity of its own. H-2 specificity is present on the surfaces of cells whereas the specificity of the protein (“antigen 3”) is not exposed on the cell surfaces. Loss of activity on freezing and thawing, freeze drying, ultrasonics, and treatment with sodium dodecyl sulphate, Tween-20 (a non-ionic detergent), or deoxycholate, is consistent with lipid specificity and more difficult to associate with protein or carbohydrate structures, since the component parts of lipids are not firmly linked as are amino acids or sugars in polypeptides or polysaccharides. Sugars in the lipid might well be susceptible to periodate destruction, indeed lipids are sometimes readily attacked by periodate in the absence of sugar residues. It is felt, therefore, that either the lipid itself carries the specificity, or that
the lipid contributes in some way to the configuration of the protein, so that both parts are necessary. It would be admirably consistent with the dramatic effects, e.g. of runt disease, for the point of attack to be the membraneous structures of the cell which carry the ribosomal synthetic machinery.

It will be of the greatest interest to test the products obtained using transplantation, enhancement and haemagglutination inhibition tests for each of these three activities, because certain differences undoubtedly exist between the T, E and H antigens so far prepared. E antigens can be frozen or freeze-dried with impunity whereas T and H antigens can not. T antigen is stable to ultrasonics whereas H-2 antigen is labile. E antigen can be lipid-extracted but this destroys activity of H-2 preparations. These facts do not necessarily weigh against the view that the same specificity is involved as one can imagine that differences in state of aggregation or degradation might markedly affect the nature of the immune response of an animal. A recent observation (Hašková and Hilgert, 1961) that a hastened rejection of skin grafts in mice can be obtained by immunization with ascitic fluid is consistent with results (to be published) indicating that the material described here does indeed carry T activity.

The H-2 specificities found to be carried by the products described agree with the known distribution of H-2 alleles in the mice used. It is not clear, however, if the products represent families of closely similar molecules each carrying one specificity or whether the several specificities belonging to one mouse strain reflect different structural features of one molecular entity. Studies in other branches of immunochemistry would suggest the latter as much more likely but a genetic basis of pseudo-alleles, where crossovers sometimes occur, does not permit one to discount the first possibility.

An unexpected finding has been the presence of H-2–C and H-2–F specificity in the product of the "non-specific" LAN tumour, which has been used as a source of presumed serologically
inert material for comparison with the active products of other tumours. The material was indeed inactive in H-2–K and also H-2–D³EK systems, but other specificities have not been tested. The ability of this tumour to grow in all kinds of mice in the face of H-2 incompatibility is of some interest. Mice can be immunized against this tumour, e.g. BALB/c mice immunized with BP8/C₃H remain susceptible to CL2 but are resistant to LAN.

The necessity of using fresh (i.e. not freeze-dried) material for extraction and all activity studies is a great inconvenience but in spite of the lability of the material, suspensions, after sterilization, have been kept for many months without detectable loss of activity.

The information obtained so far is in substantial agreement with the views recently expressed by Herzenberg and Herzenberg (1961) that H-2 activity might be associated with lipoprotein derived from the membrane fraction of cells, but the product isolated from ascitic fluid has not shown any indication of structure when examined in the electron microscope.

**Summary**

(1) Mouse H-2 antigens have been extracted from the products of ascites tumours, and an increase in activity of at least 500-fold was obtained by a simple fractionation procedure.

(2) Activity has been measured by inhibition of the haemagglutination induced with mouse anti-mouse sera by the dextran-normal human serum technique.

(3) The product is a lipoprotein; information about its homogeneity has been difficult to obtain but evidence for impurities has been sought but not found. The proportions of lipid and protein vary between 30 and 50 per cent lipid with 70 and 50 per cent protein.

(4) The protein component has species specificity and is part
of the membraneous structure of mouse cells. There are indications that H-2 specificity is associated with the lipid component.

(5) The H-2 specificities of the products obtained from different inbred mouse strains are those expected from the known distribution of H-2 alleles.

(6) Attention has been drawn to differences in some of the known properties of transplantation, enhancing and histocompatibility antigens.

REFERENCES


DISCUSSION

Kandutsch: As I understand it, your antigenic material isolated from ascitic fluid is a cell product and is insoluble or particulate. When you do agar gel diffusion tests with this and get a line, how do you exclude the possibility that your insoluble antigen has simply not left the cup, and that the line you see is due to an impurity? I ask this because when we attempted to carry out similar gel diffusion tests with our enhancing (or "promoting") preparations we found that we had a line with the rabbit antiserum (sometimes two) until I got the preparation as pure as I was able to get it, and then we got no lines. The material just remained in the cup in the system we used.

Davies: We cannot rule out completely the possibility that the antigen remains in the cup and that the line is due to impurity. However, when we worked with bacterial antigens we found that the most apparently insoluble substances would still give a diffusion line if you waited long enough; there we had other evidence that the bacterial antigen itself was responsible for the line. There is not as good evidence here, but our method of estimating this activity is by dilution on diffusion plates, and the amount of reaction we get with this product of mine is as good as the best preparation of Dr. Haughton's species-specific "antigen 3". So it must represent a fairly large proportion of this product. By all the means we have tried so far we have not been able to pick up impurity, but our evidence for physical homogeneity is rather poor.

G. Klein: Since you are using an isologous ascitic tumour system, I wondered whether it is really certain that the H-2 antigens come from the tumour cells rather than from the host, since the fluid is coming from the host. Perhaps you could clarify this by using an ascitic tumour homograft which does grow in spite of homograft reaction in a non-specific fashion. There are such tumours available.

Davies: The analogous product of a non-specific tumour didn't give any inhibition at all in the tests I showed. Full details are given in the published version of my paper. However, with one supposedly non-specific tumour (LAN) recently we have found that it does carry some specificity—it inhibits in H-2^e and H-2^f. So this is rather interesting
that it grows so readily in all kinds of mice, in some cases in the presence of this incompatibility. However, for syngeneic specific tumour systems, the yield of purified antigen is related to the weight of cells recovered from the fluid, and not to the weight of material remaining in the fluid after the cells have been removed.

G. Klein: If the antigens come from the tumour cells, then the question is whether they are released due to lysis or by secretion. This could perhaps be clarified by using different ascitic tumours that have very different tendencies to lyse. If it could be shown that it is not lysis but secretion, it may be very interesting to find out whether different types of tumour cells show differences in their secretory ability.

Hašek: I am inclined to believe that it is lysis. V. Hašková and I. Hilgert (1961. Folia biol. (Prague), 1, 81) have some evidence of increasing content of deoxyribose in the ascitic fluid with the growth of the tumour used in their experiments (sarcoma I) which might reflect the cell disintegration.

Davies: In my fractionation traces of nucleic acid are removed, but Hilgert’s correlation of DNA increase with greater antigenic potency is very suggestive of the antigen being a product of cell lysis. We also find a relatively greater yield of antigen from leukaemic cells, which are much more easily lysed than the other kinds of ascites cells we have used. If the lysis could be increased we might get a much better yield of antigen, but complete cell disruption is to be avoided because the starting material is then so much more complex.

Simonsen: I should be very interested to know where this soluble antigen of ascitic fluid would go in the isogenic host. Is it possible to label it with some radioactive label so that you can see what happens to it when no antigenic disparity is involved? Where does it go to, and what is the lifespan of this material when there is no antibody reaction against it?

Davies: We worked out that it would be prohibitively expensive to label with $^{14}$C by feeding the mice, but it might be possible with $^{32}$P.

Brent: You mentioned that you obtained haemagglutination inhibition with 50 µg. of material per ml. of antibody. I wasn’t quite clear about the strength of the antibody used.

Davies: No, 50 µg. is the amount of material which will inhibit in the smallest number of haemagglutinating doses which we dare to use
and still get a positive control. Our best materials run down to 2 or 3 μg.

Brent: It would be interesting to know whether the production of these antigens is specific to tumour cells or whether they also occur in normal tissue cells. One way of doing this would be to examine peritoneal exudate cells of normal mice for the presence of the antigens which you extract.

Davies: We use tumour cells purely for convenience, because we can get so much antigen; we can get a kilogram dry weight of cells from 5000 mice, which makes a lot of things practicable which are otherwise impracticable. I have no doubt that one could get the material from other cells; it is present, for example, in “L” cells grown in tissue culture. We have tried to get ascitic fluid in other ways, by some non-specific irritation, but not with a great deal of success; though I would not expect to find activity in such ascitic fluid. We have taken normal mouse serum through the same procedure, and again one gets an insoluble fraction in the same position in the table, but this of course is quite inactive.

Woodruff: If it were considered safe to see if you could demonstrate antigens in human beings (and I think there are circumstances in which you could do this) it would be interesting to compare ascitic fluid in people with disseminated tumours throughout the peritoneal cavity and ascitic fluid in conditions like cirrhosis of the liver.

Davies: I don’t feel we’ve got far enough to embark on such a project; we would like to learn more about this mouse material first. However, if one found the analogous human product, just by looking for the same substance isolated in the same way, the specificity of this might give a clue as to what the transplantation antigen is in man.

Woodruff: Can you produce non-neoplastic ascites in mice by making them cirrhotic, for example by giving them alcohol?

Davies: A number of papers describe the production of non-specific, non-tumour-induced ascitic fluid in mice, but we have had much less success than the authors of these papers have claimed—an occasional mouse produces something, but we haven’t had enough to put it through the mill.

G. Klein: Perhaps the best way to produce a comparable ascites of the desired quality in large quantities would be to inoculate an ascites
tumour of the antibody donor genotype; then of course any antigens secreted by the tumours just wouldn’t matter.

Davies: We haven’t any non-specific tumours derived from the mouse strains we use.

G. Klein: What is your antibody donor genotype?

Davies: We use C3H, BALB/c, ST/A and C57, and then we use all the various combinations of these, and their hybrids.

G. Klein: There are some C3H ascitic tumours growing non-specifically in foreign strains. We would be glad to send them to you.

Eva Klein: It would also be possible to use specific tumours by pre-irradiating the foreign recipients.

Davies: I would be very pleased to have your help in this matter.

Medawar: Dr. Davies, you refer to your product as antigenic, but I don’t recollect what tests you applied. Do you mean that it excites the formation of iso-haemagglutinins?

Davies: Yes, just that. For example, if we inject the C3H product into BALB/c mice we get a haemagglutinating serum which can be specifically inhibited only by the antigen injected, or by one having an H-2 specificity in common with it.

Medawar: Did you test for lysins?

Davies: No.

Simonsen: By which route did you inject?

Davies: Subcutaneously.

Medawar: Among the differences between the activities of your product and our own very much more impure one, you referred to different resistances to drying and to ultrasonics. I don’t think the tests with ultrasonics are comparable, because they were done under very different conditions. We expose whole cells suspended in water to ultrasonic irradiation, and one knows, from analogy with the stability of DNA and DNA protein, that material which would be degraded in purer form may not be degraded when still in a “structural” form. But the drying is a bit puzzling. We dry our material from a suspension in water which is very rapidly frozen, and it is dried at a temperature which isn’t allowed to rise above — 5° C; our preparation treated in this way retains, not all activity, but a high proportion of activity. Did I understand correctly that your preparation loses all its activity on freeze drying?
Davies: No, we don’t lose all the activity by any means, and what we lose may only reflect the poor dispersion of the freeze-dried product on resuspension. With regard to ultrasonics, it seems possible that you could lose a great deal of activity without detecting the loss by your test. In my test a one-tube difference in titre is a 50 per cent change in activity, and from successive ultrasonic treatments, sampling after each, I can plot a curve for loss of activity. However, the ultrasonic effect may be only a feature of the test system and is not necessarily an intrinsic loss.

As for the freeze-drying: Dr. Kandutsch, you can freeze-dry your preparations and they retain activity, don’t they?

Kandutsch: Yes.

Davies: And if you extract with solvents the activity is also retained?

Kandutsch: This is not so clear-cut. With some solvents under some conditions, activity is retained. I haven’t investigated enough solvents or conditions to make a definite statement on this.

Davies: And your materials, Prof. Medawar? With some solvents you retain some activity?

Medawar: Yes, after extraction with lipid solvents we get this residue activity which we suspect is not H-2 activity at all.

Davies: On extracting our material with ether at $-15^\circ$ we can get back a residue that retains some activity; it is a lot less active, but then it is also a lot less dispersible. When we extract with chloroform–methanol, also in the cold, activity is lost.

Amos: Do you get precipitin activity with isoantibodies with this material?

Davies: I thought it would be most unlikely, and we haven’t tried. It will precipitate with rabbit antisera but I take this to be due to association of H-2 with the species-specific “antigen 3”.

Amos: It is usually said that mouse antibodies do not precipitate, but they can with the right sort of antigen. Mouse anti-mouse thyroid will give good precipitin reactions.

Another point concerning absorption of anti-E, you can’t relate the anti-EK to anti-E. This must be non-specific absorption, because the anti-K should still have given you agglutination. You had two examples, one where you thought this might be absorption with F and one with E. In both of these you had an antibody with a double specificity;
the other components should not have been affected by the presence of the inhibitor.

Davies: There is only one thing in common there. For example, when I had $D^kE^K$ serum, I got very good inhibition with the homologous (in the true sense) substance. Now if I have a material extracted from mice which carried some other H-2 specificities but had $E$, but not $D^k$ or $K$, then I get a lesser degree of reaction.

Amos: What were the test cells?

Davies: The test cells were $D^k$, $E$ and $K$ and were from the mouse which provided cells for immunization. So we were picking up all three things there. In my Table V they were monospecific sera, or at least very nearly so, but in my Table IV we were testing for several characters, and I believe they are genuine cross-reactions. Where you say anti-$K$ should still have agglutinated, it would probably not do so because we have diluted the antibody to the extent that the homologous cells are only just agglutinated, in order to achieve maximum sensitivity.
AN ISOANTIGENIC LIPOPROTEIN FROM SARCOMA I*

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Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

The earliest investigations into the nature of the transplantation isoantigens responsible for the phenomenon of immunological enhancement were carried out by Snell (1952) and by Day and co-workers (1954). We have continued to rely chiefly upon the enhancement test as a measure of tissue isoantigens and, in more recent stages of this work, use has been made of a haemagglutination inhibition assay. The enhancement phenomenon is dependent upon the production in host mice of circulating antibodies specific for the isoantigens present in a tumour homograft (review by Snell, 1957). The enhancement assay is, therefore, a measure of complete antigens. In contrast the haemagglutination inhibition assay may be expected to show a positive test with haptenic or non-antigenic fragments as well as with complete antigens. The inhibition test also provides a means for assaying the activity of antigen specificities which are shared by the particular donor and recipient strains (A and B10.D2—an H-2^d subline of C57BL/10) used in the enhancement test, while the enhancement test measures only the activity of antigens present in the A strain but absent from B10.D2. The enhancement assay was carried out as described previously (Kandutsch and Reinert-Wenck, 1957). The haemagglutination-inhibiting properties of different preparations were determined by means of the polyvinylpyrrolidone

* Supported by a grant from the American Cancer Society (E-26) and by Research Grant C-1329 from the National Cancer Institute, United States Public Health Service.
(PVP) test (Stimpfling, 1961). The use of PVP as a developing agent is necessitated by the incomplete character of haemagglutinins in mouse isoantiserum. Varying amounts of each preparation were added to a constant quantity of antisera and incubated for two hours at room temperature followed by an additional 18 hours' incubation at 4°C. The absorbed antisera were then titrated against erythrocytes collected from appropriate strains. All titrations were done in triplicate and the mean log of the reciprocal titres was determined.

As preliminary steps towards the isolation of the antigens our initial efforts were directed towards determining the distribution of the antigens and their stability to a variety of factors which might be applied in an isolation procedure or provide a clue to their identity. The results of these experiments led us to conceive of the antigens as molecules formed in some part of an essential, labile protein, possibly with carbohydrate as a second component, localized in the membranes of cells, and most concentrated in tumour (sarcoma I), spleen, and parotid glands (Kandutsch and Reinert-Wenck, 1957; Kandutsch, 1960). Further investigations carried out in the light of this information have led to the isolation of an isoantigenic lipoprotein which, in studies conducted so far, appears to be electrophoretically homogeneous.

**Isolation procedure**

The isolation procedure shown in Fig. 1 can be divided into three phases.

(A) **Preparation from sarcoma I (ascites form) of a particulate fraction which may consist principally of cellular membranes** (Kandutsch, 1960). Initially the procedure for obtaining a membrane fraction was essentially that used by Smith and co-workers (1957) to obtain a membrane fraction from liver cells. The procedure has been rather extensively modified to omit repeated extractions with highly concentrated salt solutions.
(B) Extraction of antigenic activity from the particulate fraction. In a previous publication, extraction of antigenic activity by a 5 per cent solution of Triton, pH 9, at room temperature, was reported (Kandutsch, 1960). We have since found that the yield of antigenic activity in the extract is much higher when extraction is carried out for from 10 to 14 days at refrigerator temperatures at a pH maintained at about 7.5 by the addition of tris (tris(hydroxymethyl)aminomethane). After the extraction period the mixture is centrifuged at 105,000 g for one hour. Material is recovered, free of Triton, from the supernatant fraction by precipitation with ten volumes of cold acetone followed by an

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WASHED SA. I (ASCITES) CELLS

<table>
<thead>
<tr>
<th>Homogenize in dist. H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuge - discard supernatant fraction.</td>
</tr>
</tbody>
</table>

SEDIMENT

<table>
<thead>
<tr>
<th>Homogenize in dist. H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digest 1.5 hrs. at 37 °C with DNase (5 mg/100 ml), RNase (12 mg/100 ml) &amp; 0.025M MgCl₂</td>
</tr>
<tr>
<td>Centrifuge - discard supernatant fraction.</td>
</tr>
</tbody>
</table>

SEDIMENT

| Wash 1x with 0.14 M NaCl and at least 3x with dist. H₂O |
| Freeze-dry |

PARTICULATE FRACTION

| Homogenize 1 gm in 40 ml 5% Triton. Add a crystal of Thymol and allow to stand 10-14 days at 0-4 °C maintaining pH at 7.5 with Tris. |
| Centrifuge at 105,000 xg for 1 hr. - discard sediment. |

SUPERNATANT

| Pour into 10 vols. Acetone (-20 °C) |
| Centrifuge - wash 2x with ether |

SEDIMENT (Whole Triton extract)

| Extract 2x with 0.1 M phosphate pH 7.2, 4x with 0.14 M NaCl |

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SUPERNATANT FRACTION

(Triton Soluble Lipoprotein TSL)

(Water Soluble Fraction of Triton Extract)

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Fig. 1. Isolation of Triton-soluble lipoprotein (TSL) from Sarcoma 1 (ascites form). Unless otherwise indicated, centrifugation was for 30 minutes at 60,000 g.
ether wash. Between 20 and 40 per cent of the weight of the particulate fraction is recovered from the Triton extract.

(C) Fractionation of the acetone-precipitated material by extraction with aqueous salt solutions. Three extractions with 0.1M-phosphate buffer pH 7.2 and three extractions with 0.14M-NaCl dissolve approximately 85 per cent of the material. Only small amounts of

![Graph](image)

**Fig. 2.** Inhibition of the haemagglutinating activity of two isoantisera, C57BL/10 anti-B10.D2 (A) and (C57BL/6 × DBA/2)F1 anti-C3H (B), by increasing quantities of the different fractions. Blood cells used in the assay were collected from A/Sn mice. Cells for negative control tests were from strain B10.D2.

- Particulate fraction
- Whole Triton extract
- Triton-soluble lipoprotein (TSL)
- Water-soluble fraction

protein are obtained in further extracts. The fraction poorly soluble in water appears to be an electrophoretically homogeneous lipoprotein and is hereafter referred to as Triton-soluble lipoprotein (TSL). Antigenic activities of the Triton extract and of subfractions obtained by differential extraction are compared in Table I, and their relative abilities to inhibit haemagglutination are illustrated in Fig. 2.
It is apparent that most of the antigenic activity present in the whole Triton extract was concentrated in the TSL fraction. The fraction of the whole Triton extract that was soluble in water possessed only low levels of activity. Antibody titres in treated animals were positively correlated with enhancement of tumour homografts in the same animals. Similar results were obtained in a number of other enhancement assays of fractions comparable to those shown in Table I although the percentage of mice

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Mg. injected per mouse</th>
<th>Enhancement test (No. dying out of 10)</th>
<th>Geometric mean of haemagglutinin titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Triton extract</td>
<td>7.5</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>TSL</td>
<td>7.5 equiv.</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Water-soluble fraction</td>
<td>7.5</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Graft: Sarcoma I (solid)

Whole Triton extract 15.0 9 1/80
7.5 4
TSL 15.0 8 1/80
7.5 3
Water-soluble fraction 15.0 2 <1/5
7.5 0
None 0 <1/5

* The amount obtained from 7.5 or 0.75 mg. of whole Triton extract

In the enhancement test, the number of mice dying as a result of the growth of the tumour homograft is proportional to the amount of antigen injected. Preparations were injected intraperitoneally in three equal doses at 4-day intervals into mice of strain B10.D2 (an H-2d subline of C57BL/10). Sarcoma I was inoculated subcutaneously 10 days after the last injection. The derivation of the fractions tested is described in Fig. 1. Antibody titres were determined on individual sera obtained from 5 mice, 7 days after the last injection.
dying at a given dose varied among experiments. Such variation in the dose of antigenic material required to produce enhancement has been observed throughout these studies and is presumably due chiefly to variations in the growth characteristics of the tumour. When the ascites form of sarcoma I was used as the challenging graft, the amount of antigenic material required to produce enhancement was considerably less than when the solid form of the same tumour was used. However, the use of the solid tumour afforded better quantitation of antigenic activity. In a number of enhancement assays, the whole Triton extracts and the TSL fraction were at least as active as the particulate fraction from which they were derived and in some experiments they appeared to be considerably more active on a weight basis. Results comparable to those shown in Table I were obtained with the haemagglutination inhibition assay except that the whole Triton extract appeared to inhibit only slightly. Possibly the haemagglutination inhibition assay provides a more quantitative picture of the distribution of activity since the TSL fraction which contained most of the antigenic activity constituted only about 15 per cent of the whole Triton extract.

Because of the low levels of activity present in the water-soluble fraction of the Triton extract and its complexity—at least eight components were indicated by chromatography on diethylaminoethyl (DEAE)-cellulose columns—further studies were concentrated on the Triton-soluble lipoprotein.

**Properties of the Triton-soluble lipoprotein**

Different preparations of freeze-dried TSL were powders ranging in colour from tan to brown. It dissolves readily in a 1 per cent solution of Triton at pH 7 or above, in concentrations as high as 80 mg./ml., to give an optically clear solution. 

*Electrophoresis:* Some electrophoretic patterns obtained with a Tiselius apparatus are shown in Fig. 3. Triton was included in
each of the buffer systems and the difference in the concentration of Triton between the sample and the buffer appears in the patterns as a sharp spike following the lipoprotein peak. This boundary migrates at a rate not markedly different from that of the lipoprotein. The TSL migrated as a single component in the two

![Diagrams of electrophoretic patterns](image)

**Fig. 3.** Tracings of electrophoretic patterns of TSL. Freeze-dried TSL was dissolved in buffer solution containing 1 per cent Triton and dialysed overnight *versus* the same buffer solution. The concentration of TSL was 1.3 per cent and 1 per cent in barbitone and phosphate buffers, respectively. Field strength was 13 volts/cm.

buffer systems shown and also in tris buffer of ionic strength 0.1, pH 7.5. An attempt to examine its electrophoretic characteristics at pH 5 in acetate buffer was unsuccessful since the lipoprotein was precipitated from solution under these conditions. Estimations of the isoelectric point from the limited mobility data obtained so far suggest that it falls within the pH range 4 and 4.2, a range
where destruction of enhancing activity has been shown to occur (Kandutsch and Reinert-Wenck, 1957). Electrophoresis on filter paper or cellulose acetate paper, in a variety of buffers over a pH range from 6·2 to 9, whether Triton was included or omitted from the buffer in the electrode compartments, gave results similar to those obtained with the moving boundary apparatus—only a single component being visible. Attempts at electrophoresis in starch gel met with poor success since the substance trailed from the origin showing a diffuse band.

*Chemical composition:* The results of some chemical analyses of TSL are shown in Table II. Since the methods used to determine

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Method</th>
<th>Percentage of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose</td>
<td>(Winzler, 1955)</td>
<td>1·8</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>(Warren, 1959)</td>
<td>0·13</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>(Rosenlund, 1956)</td>
<td>0·6</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Semi-micro-Kjeldahl</td>
<td>14·9</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>(King, 1932)</td>
<td>1·0</td>
</tr>
<tr>
<td>Lipid</td>
<td>Extracted with 2:1 CHCl₃: methanol</td>
<td>25·8</td>
</tr>
</tbody>
</table>

hexose, sialic acid and hexosamine are not entirely specific, the values shown for these compounds may be erroneously high. It is clear that carbohydrate constitutes at best only a small portion of the lipoprotein. Lipid extracted with 2:1 CHCl₃: methanol constituted 25·8 per cent of the weight of the TSL and most of the phosphorus present in the TSL was found in the lipid extract. The concentration of phosphorus in the residual protein was only 0·18 per cent as contrasted to 1·0 per cent in the TSL before extraction. The concentration of nitrogen in the residual protein (14·7 per cent) was similar to that found in the whole lipoprotein. The ultraviolet absorption spectrum of TSL (Fig. 4) shows a peak due to protein in the region of 280 mμ and an additional absorption band in the region 230–260 mμ. The latter band is probably
due, at least in part, to the partially auto-oxidized lipid moiety (see Gurd, 1960, for a summary of effects of auto-oxidation on absorption spectra of lipoproteins). Auto-oxidation of the lipid moiety is also the most likely explanation for the brown colour of the lipoprotein which has been observed to increase in intensity with time and handling.

Modification of TSL by snake venom

Although TSL appears to be an electrophoretically homogeneous substance, it seems possible that the entire, intact molecule may not be required for antigenicity. In view of this, and because specific modifications may be expected to throw further light upon the structure, and the nature of the antigenic
sites, of the lipoprotein, we have begun to investigate ways in which the structure of TSL can be modified, and the effects of structural modifications on activity as measured by the enhancement and haemagglutination inhibition tests. Structural modifications that increase the solubility in water of the lipoprotein can be determined conveniently by measuring changes in the turbidity of a suspension of the lipoprotein. TSL, suspended by homogenization in 0.1M-tris buffer, pH 7.9, at a concentration of 0.1 mg./ml., gives a turbidity reading of 0.15 (measured as absorbancy at 660 μm in a 1 cm. cell in a Beckman DB spectrophotometer). The effects of some enzymes on the turbidity of such a suspension are shown in Fig. 5. The addition of trypsin or chymotrypsin to the suspension produced a rapid but limited decrease in turbidity. Addition of lipase produced a relatively slow decrease in turbidity which also approached a limiting value while considerable turbidity was still evident. Turbidity decreased rapidly in the presence of snake venom and the reaction proceeded to the point where the suspension appeared optically clear after approximately one hour. Boiled venom was inactive, suggesting that the active factor may be an enzyme. Pepsin had no effect on turbidity under the conditions used.

In view of the marked solubilizing effect of snake venom it is of interest to examine further the immunological and chemical properties of the altered TSL. Because of the limited amount of TSL available only the preliminary studies described below have been carried out to date.

One hundred mg. of TSL in three ml. of 0.1M-tris buffer pH 7.9 was digested with 2.8 mg. of snake venom for one hour at room temperature (22°). The mixture was then dialysed for five hours with stirring versus 0.1M-acetate buffer pH 5. The resulting precipitate, centrifuged down, washed twice with buffer and freeze dried, weighed 78 mg. It dissolved in 0.1M-tris or barbitone buffers (pH 7.9 and 8.6, respectively) to give a tan, slightly opalescent solution. Moving boundary electrophoresis indicated
Fig. 5. Effects of some enzymes on the turbidity of a TSL suspension. In experiment A, TSL was suspended in 0.1 M-tris-maleate buffer pH 7.3. In experiment B, the buffer was 0.1 M-tris, pH 7.9, except that 0.1 M-phosphate, pH 6.6, was used when digestion was with pepsin. The concentration of TSL was approximately 0.25 mg./ml. Concentrations of enzymes were: trypsin (crystallized), 0.1 mg./ml.; α-chymotrypsin (from ethanol), 0.1 mg./ml.; pepsin (crystallized), 0.2 mg./ml.; lipase from porcine pancreas (Cal-Biochem., B grade), 0.2 unit/ml.; and lyophilized snake (Crotalus adamanteus) venom from Ross Allen Reptile Institute, 0.08 mg./ml.

the presence of a major component and a small amount of a second component (Fig. 6). In phosphate buffer pH 6.3, only one component (mobility 4.06 x 10^{-5} \text{cm}^2 \text{sec}^{-1} \text{volt}^{-1}) was evident. The mobilities of both components at pH 8.6 were considerably
different from those found for TSL in the presence of Triton. However, since Triton may influence the mobilities, no conclusions can be drawn from such a comparison. The minor component in Fig. 6 may therefore represent a minor contaminant of TSL not detected by electrophoresis in the presence of Triton. It is, however, at least as likely that the minor component in Fig. 6 may represent that portion of TSL not altered by the venom enzyme, or some venom protein co-precipitated with the altered

TSL. Estimated from its mobility at pH 6·3 and 8·6 the isoelectric point of the major component was essentially the same as that for unaltered TSL. As shown in Fig. 7, venom-digested TSL appeared to inhibit red cell haemagglutination somewhat more effectively than did the unaltered material.

Although much more work is required to establish fully the purity and structure of the TSL and of the more soluble derivative produced by digestion with snake venom, it seems probable that these goals will be reached. The isolation of these substances, however, raises further questions concerning the relationships of their molecular structures to the minimum structural features
required for each of the various immunological effects which transplantation antigens are able to cause. Since, as appears certain, transplantation isoantigens are components of the lipid-protein (and possibly also carbohydrate) complex which forms cell membranes, it is likely that the size and chemical complexity of an isolated antigenic molecule may be related to the methods used to dissociate the membranes into component parts. TSL, isolated by the relatively mild methods used, may thus contain a relatively simple antigenic segment within a larger, more complex, molecule. The conversion, by snake venom, of TSL into a water-soluble substance which yet retains its capacity to inhibit certain specific haemagglutinating reagents indicates that the intact TSL molecule may not be required for production of this effect. On the other hand, it has been suggested that subsi-
diary attachments to a substance of genetically determined specificity may influence the occurrence and nature of the immunological response incited in vivo (Snell, 1957; Kandutsch, 1961). The relationships of antigen structure to the kind of immunological response obtainable, along with investigations into the chemistry of genetically determined alterations in the antigens and the roles of these substances in cell membranes, may be fertile fields for further research.

**Summary**

A Triton-soluble lipoprotein isolated from a particulate fraction of sarcoma I appeared to be homogeneous in moving boundary and zone electrophoretic studies. It was isoantigenic, as measured by the enhancement test or by its ability to induce the formation of red blood cell agglutinins, and was active in a haemagglutination inhibition assay. The Triton-soluble lipoprotein was degraded to a more water-soluble form by digestion with snake venom. The altered substance, recovered by acid precipitation, contained two electrophoretically separable components—one in small amount. The altered material retained the ability to inhibit red blood cell agglutination by specific antibody.

Some of the chemical and physical properties of the Triton-soluble lipoprotein and of the material recovered after digestion with snake venom are described.

**Acknowledgement**

We are grateful to Dr. Eugene Roberts for suggesting that the effects of snake venom be examined and for a supply of the venom.

**REFERENCES**


DISCUSSION

Medawar: Do you know that your preparation doesn’t sensitize against, for example, skin grafts? I ask this because sarcoma I is, as I understand it, an easily “enhanceable” tumour. Secondly, do you attribute your results to the formation of humoral antibodies—that is to say, can you passively transfer your effect with serum? This would definitely make it an enhancement phenomenon.

Kandutsch: I can’t answer either of these questions affirmatively with regard to my purest preparations. However, I think Dr. Hašková has found that preparations similar to those I used, and which I expect would produce the effects that I find, do also produce sensitization to a skin graft. We haven’t had enough material yet to investigate this fully. With regard to the enhancement phenomenon, we have not tried to transfer this tumour-promoting effect with serum from animals immunized with the purest material but the test has always responded to antiserum from animals immunized with less pure preparations up to this point, and I had not thought it really necessary to try it again.

Davies: I think Gorer said that sarcoma I lacked several of the H-2 antigens of A mice. I wondered if that might affect your system.

Amos: It depends on the sarcoma I; there are great differences between sublines of sarcoma I.

Kandutsch: We have used this ascites form for several years, and it has become increasingly malignant. One has to use quite a lot in order to
enhance to the solid form now, so that it seems that at least some quantitative differences between these two forms of the same tumour have developed.

_Eichwald_: I have a question regarding the assay system in which you use the promotion of tumour growth. You express your results as the number dying out of 10 mice. If a mouse dies before the end of your critical period how do you count that in your final fraction of 10 mice? This may seem rather petty, but since the groups are fairly small, a difference of one or two mice may make a great deal of difference.

_Kandutsch_: In these particular experiments no mice died from causes other than the growth of the tumour. However, sometimes it happens that one mouse dies from some unknown cause before the period when the tumour would kill them, although this tumour kills in about two weeks, or three at the most. In this case I have usually expressed it as a percentage, even though it seems a little extreme when you have only 10 mice.

_Feldman_: I was very interested in your application of snake venom to this antigenic preparation, so that you are able to change the physical properties of your material without apparently changing the antigenic behaviour. I believe snake venom has a high content of lecithinase. Did you try to see whether there is lecithinase activity in your snake venom preparations, and whether its action could explain your results?

_Kandutsch_: At least one procedure for isolating lecithinase from snake venom has been reported, but the venom used is from a tropical rattlesnake rather than the Eastern diamond-back, which is the one I had. When I tried to apply that procedure to my snake venom I obtained a fraction but it did not produce the effect I had observed with whole venom. I haven’t yet really examined this to see whether it has in fact lecithinase activity. Snake venom has, besides lecithinase activity, several proteolytic enzymes, diphosphoesterases and perhaps many more.

_Davies_: There is undoubtedly lecithin in the lipid fraction of my material. Lysolecithin is the product produced by snake venoms from lecithin, and when I add lysolecithin to my material it also clarifies the solution, but with loss of activity.

_Kandutsch_: My material, once it has been digested with the snake venom, undoubtedly still has a fairly large lipid portion attached to the
protein; it will still stain with lipid stains, for example. So, whatever the snake venom removes, it does not remove all the lipid.

Davies: I am puzzled by one thing. You gave the nitrogen content of your Triton-soluble lipoprotein as 14.9 per cent, but you have 26 per cent extractable lipid, and if that is nitrogen-free it puts the nitrogen content of the protein up to 20 per cent, which is impossible. Is there some nitrogen in the lipid?

Kandutsch: There is some nitrogen in the lipid, but I have not been able to get a satisfactory analytical value for it.

Davies: It is just possible that the second component you get on electrophoresis after treatment with snake venom might be lysolecithin. We have had trouble with ultracentrifuging solutions of our material with lysolecithin, because lysolecithin forms micelles in water, and the micelles sediment at about the rate of serum globulin in the ultracentrifuge, giving an apparent molecular weight of about 150,000.

Kandutsch: Wouldn’t lysolecithin dialyse out?

Davies: I don’t know. I think it might not, because it does form these large micelles and is therefore not distributed in solution in the ordinary sense.

Medawar: Dr. Kandutsch, what is your present feeling about the rôle of carbohydrate in these antigens, and the inactivation produced by sodium periodate?

Kandutsch: The amounts of carbohydrate that we find in these preparations are very small, and I can’t really say that the effect of periodate is on the carbohydrate. Periodate is known to split amino acids such as threonine or serine if they are on the N-terminal end of a protein. We have done some qualitative studies of the amino acid composition of the Triton-soluble lipoprotein, and we have a quantitative study under way and there does seem to be quite a lot of threonine and serine in these molecules. Many of the lipoproteins in serum have serine and threonine end groups, so this is a possibility. It would be very interesting if one could simply knock off the N-terminal amino acid and thereby destroy the antigenic activity, thus localizing an antigenic site.

Davies: Periodate, of course, can attack lipids rather readily; certainly some polyhydroxy lipids are just as susceptible to periodate destruction as sugars. Dr. Marjory Macfarlane described a serologically active lipid which contained no sugar, but was destroyed by periodate.
Kandutsch: The lipid that is attached to this material is obviously very labile to auto-oxidation in air, and I am sure that periodate would oxidize it also. As far as I know, however, this auto-oxidation of the lipid has not appeared to influence the activity of the preparations very much, but then I don’t have a very quantitative test, and perhaps I could miss a sizeable change.
STUDIES ON CHEEK POUCH SKIN HOMOGRAFTS IN THE SYRIAN HAMSTER*

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The existence of "privileged" or favoured sites in the body, in which grafts of living tissue of foreign origin may long be accepted by the host, apparently exempted from the usual immunologically procured rejection process, is well established. Familiar examples are the brain, the anterior chamber of the eye, the substantia propria of the cornea, and possibly the testis (see Medawar, 1948; Billingham and Boswell, 1953; Russell, 1961). The uniqueness of each of these sites seems to depend upon the fact that the physiological pathways necessary for either the evocation or the putting into effect of an immunological response are incomplete in some respect—i.e., there is a break in either the afferent or efferent pathways of the immunological reflex.

The multiplicity of reports of the successful transplantation of tissues, normal and malignant, of both homologous and heterologous origin, to the cheek pouch of the Syrian hamster (Mesocricetus auratus), where they rapidly acquire a rich blood supply and may thrive for considerable periods (see Lemon et al., 1952; Cohen, 1961; Yohn et al., 1962), constitute a strong prima facie case that this, too, is an immunologically privileged site.

Confirmatory evidence of a more direct nature has been forthcoming from studies in which the fates of skin homografts or skin heterografts implanted into the connective tissue of the wall

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of the cheek pouch have been compared with those of similar grafts transplanted orthotopically to recipient areas prepared in the skin of the trunk (Billingham, Ferrigan and Silvers, 1960; Cohen, 1961). Furthermore, it has been shown that the privilege extended to foreign grafts in the pouch is not absolute: homografts undergo prompt destruction if implanted into the pouches of previously sensitized hosts, and the lives of long-established homografts in the pouches of normal animals are soon terminated if their hosts are sensitized by means of orthotopic skin homografts from the original donor strain.

In the course of studies designed to elucidate the basis of the privileged status of the cheek pouch, the fate of homografts of cheek pouch “skin”, transplanted to the environment of normal skin, has been investigated. A preliminary account of this work and some implications of the findings constitute the subject matter of this paper.

**Materials and methods**

Adult hamsters belonging to three different, partially inbred strains of remote common origin, M.H.A., C.B. and L.S.H., were employed. Although none of these strains as yet can properly be designated as isogenic, each is completely uniform with respect to its histocompatibility genes—i.e., it is isohistogenic (Billingham and Silvers, 1959). The three donor/recipient strain combinations employed and the median survival times of medium-sized orthotopic homografts of normal trunk skin transplanted between them are set out in Table I. It may be added that hamster stocks in general are anomalous in that the number of demonstrable segregating histocompatibility genes is very small. Analyses by Billingham, Sawchuck and Silvers (1960) have shown that the present strains differ only with respect to one or two important histocompatibility loci. Nevertheless, the homograft reactions promoted by these differences are just as powerful as, and
indistinguishable from, those encountered in other species where homografts confront their hosts with a whole battery of “foreign” antigens.

**Structure and preparation of cheek pouch skin grafts**

The paired cheek pouches, whose highly vascular walls constitute the milieu into which foreign grafts are implanted, are elongate, highly distensible and evaginable diverticulae of the buccal cavity employed by hamsters for the temporary storage of relatively enormous volumes of food. The wall of the pouch consists of: (1) a stratified epithelium several layers in thickness and totally devoid of hairs and glands, firmly united to (2) a relatively dense and well-vascularized layer of rather dense fibrous connective tissue. Abundant longitudinal muscles are present below this fibrous connective tissue near to the aperture of the pouch. A fairly thick outer layer (3) of loosely packed areolar tissue unites the pouch to the subcutaneous tissue of the cheek and allows the entire pouch to be everted easily. A detailed account of the structure and anatomical relationships of these organs has been presented by Priddy and Brodie (1948).

To obtain pouch “skin” grafts the organs were everted from freshly killed animals, thoroughly cleaned with 5 per cent Dettol solution, and then excised as close to their point of union with the buccal cavity as possible. A longitudinal incision along one
side of the pouch resulted in a more or less flat sheet of skin which was immersed in normal saline solution, containing 150,000 units of penicillin and 125 mg. dihydrostreptomycin sulphate in suspension/ml., for about 30 minutes as a prophylactic measure. The slight swelling of the connective tissue which occurred in this medium facilitated trimming off excess areolar tissue necessary to ensure prompt healing after transplantation. Circular grafts, usually about 2·0–2·5 cm. in diameter, were then cut from areas of pouch skin in which muscle fibres were absent.

The grafts were transplanted to beds of appropriate size prepared in the skin of the side of the hosts’ chests according to our standard procedure for grafting normal skin (see Billingham, 1961).

Experimental findings

(1) The fate of cheek pouch skin isografts

To provide the necessary controls, 40 C.B. hamsters received grafts of C.B. pouch skin which were maintained under observation for at least 60 days. Provided that the thickness of these grafts was not excessive, they healed-in just as rapidly and satisfactorily as isografts of appropriately trimmed normal skin and no evidence of immunological rejection was obtained. As anticipated, these pouch skin grafts conserved their anatomically distinctive features. They remained highly vascular, being flush pink in colour, and their hairless epithelial surface usually displayed a characteristic pattern of fine ridges or corrugations. Furthermore, they were covered with thin semi-transparent cuticular layers which peeled off easily and were promptly regenerated—indirect evidence of a chronic high rate of mitotic activity in the epidermis.

Comparison of the diameters of these grafts two weeks after transplantation, when primary healing was complete, with their diameters after they had been in residence for 50 days indicated that most of them had undergone slight contracture, as evidenced
by a loss of 2–4 mm. in diameter. Further contracture did not occur subsequently.

(2) The fate of cheek pouch skin homografts

The survival times of pouch skin homografts transplanted between normal adult members of our strains are summarized in Table II. Although with each combination some of the homografts were rejected quite promptly as a consequence of typical acute reactions, the majority survived at least twice as long as

Table II

Survival times of homografts of cheek pouch skin exchanged between adult hamsters of different strains

<table>
<thead>
<tr>
<th>Strain combination</th>
<th>Number of animals grafted</th>
<th>20th day</th>
<th>30th day</th>
<th>40th day</th>
<th>50th day</th>
<th>100th day*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B.→M.H.A.</td>
<td>94</td>
<td>61 (65%)</td>
<td>39 (41%)</td>
<td>35 (37%)</td>
<td>29 (31%)</td>
<td>21%</td>
</tr>
<tr>
<td>C.B.→L.S.H.</td>
<td>50</td>
<td>36 (72%)</td>
<td>34 (68%)</td>
<td>33 (66%)</td>
<td>26 (52%)</td>
<td>52%</td>
</tr>
<tr>
<td>L.S.H.→C.B.</td>
<td>51</td>
<td>40 (78%)</td>
<td>26 (51%)</td>
<td>24 (47%)</td>
<td>21 (41%)</td>
<td>35%</td>
</tr>
</tbody>
</table>

* The percentages of grafts alive on the 100th post-operative day are based upon long-term observations of fairly small groups of animals.

homografts of ordinary skin of similar genetic origin (see Table I), and an appreciable proportion survived in a perfectly healthy, "isograft-like" condition for as long as their hosts remained under observation—more than 200 days in some cases (see Figs. 1 and 2). The long-delayed rejection of some of the hitherto healthy-looking homografts took place fairly rapidly, within about 7–14 days of the appearance of adverse signs, such as oedema, intradermal haemorrhages, and epithelial weakness or ulceration. With other pouch skin homografts that long outlived control skin homografts, rejection was of the drawn-out or chronic type, in which epithelial desquamation, minor scabbing and epithelial weakness with partial recovery, and progressive contracture, to
Fig. 1. Showing perfectly healthy L.S.H. strain pouch skin homograft of more than 100 days standing on the chest of a C.B. strain host.

Fig. 2. Section through L.S.H. pouch skin homograft which has remained in an isograft-like condition for more than 100 days on the chest of a C.B. strain host. Note distinctive compact cuticle and complete absence of cellular infiltration. (×110.)
produce grafts having a wart-like or papillomatous appearance, were conspicuous features. As a group, even the healthy homografts showed more tendency to contract than did the isografts. Examination of biopsy specimens from long-established, healthy-looking pouch skin homografts frequently revealed local areas of mild lymphocytic infiltration in the graft dermis which may have been indicative of a feeble, but completely ineffective, reaction on the part of the host (see also Cohen, 1961). There was no evidence that the size of these pouch skin homografts influenced their fate. The prognosis for long-term survival of very large grafts was certainly not inferior to that for small grafts.

These unexpected prolongations of survival of cheek pouch skin homografts, in the case of each of the three different donor/recipient strain combinations tested, strongly suggested that homografts of pouch skin are themselves privileged for one reason or another. Either (a) their capacity to sensitize their hosts is abnormally feeble, or (b) they are much less susceptible to a state of sensitization than skin homografts. Experimental evidence that may be considered to refute this second possibility outright includes: (1) the finding that pouch skin homografts are consistently rejected in a peremptory manner if transplanted to hosts which have previously rejected homografts of ordinary skin from the same donor strain; and (2) the fact that hamsters which have rejected homografts of pouch skin soon reject subsequent grafts of pouch skin from the same donor strain, whereas most hamsters bearing long-established, healthy grafts of homologous pouch skin will usually accept a second pouch skin homograft transplanted to the opposite side of their chest (see Table III). Two out of 18 hamsters bearing healthy primary homografts of pouch skin of at least 50 days standing rejected secondary grafts after 27 and 40 days respectively, yet their primary grafts remained healthy. This paradoxical state of affairs may simply reflect the ability of established grafts to withstand low levels of sensitization.
### Table III

**Fate of second-set pouch skin homografts**

<table>
<thead>
<tr>
<th>Status of primary graft at second operation†</th>
<th>Survival times of primary grafts (days)</th>
<th>No. of animals given second-set grafts</th>
<th>Survival times of second-set grafts (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rejected</td>
<td>&lt;20</td>
<td>11</td>
<td>≤15</td>
</tr>
<tr>
<td>Rejected</td>
<td>20–40</td>
<td>10</td>
<td>16–20</td>
</tr>
<tr>
<td>Rejected</td>
<td>41–70</td>
<td>6</td>
<td>21–25</td>
</tr>
<tr>
<td>Alive and healthy</td>
<td>&gt;50–90</td>
<td>18‡</td>
<td>26–50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

* In these tests the hosts belonged to the M.H.A. or L.S.H. strains; the donors belonged to the C.B. strain.  
† All second-set grafts were transplanted either 2–4 weeks after breakdown of the primary graft was complete, or after a host had borne its primary C.B. graft in a healthy condition for 50–90 days.  
‡ In three of these animals rejection of the second graft was soon followed by rejection of the primary graft. Two animals were unique in that, despite rejection of their second-set grafts, their primary grafts remained perfectly healthy.

### (3) Preliminary analysis of the anomalous behaviour of pouch skin homografts

Evidence that homografts of cheek pouch skin are not abnormally resistant to transplantation immunity is forthcoming from the finding that long-established, healthy, pouch skin homografts are usually destroyed within one or two weeks following: (1) transplantation to their hosts of grafts of homologous normal skin from the original donor strain, of much smaller size than the established pouch skin graft. The mode of rejection of these sensitizing grafts was that of “first-set” homografts (Medawar, 1944), indicating the failure of the pouch grafts to sensitize their hosts; or (2) the injection of the host intraperitoneally or intravenously with a relatively low dosage of homologous leucocytes—as few as 1 million were effective—prepared from the buffy coat of blood obtained from members of the original donor strain; or (3) by the intradermal injection of bone marrow cells (see Table IV). These findings leave no doubt as to the normal susceptibility of pouch skin homografts to a state of sensitization elicited by proxy. In all cases where rejection was procured in
Table IV

DESTRUCTION OF LONG-ESTABLISHED POUCH SKIN HOMOGRAFTS BY EXPOSURE OF HOSTS TO HOMOLOGOUS CELLS OR TISSUE ADMINISTERED BY VARIOUS ROUTES

<table>
<thead>
<tr>
<th>Antigenic stimulus</th>
<th>Dosage</th>
<th>Route of administration</th>
<th>Number of hosts tested*</th>
<th>Survival times of established pouch skin homografts after exposure to immunizing stimulus (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Small graft</td>
<td>Orthotopic</td>
<td>6</td>
<td>6 × 13–16</td>
</tr>
<tr>
<td>Leucocytes†</td>
<td>10 × 10⁶</td>
<td>Intraperitoneal</td>
<td>4</td>
<td>4 × 9–10</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>10 × 10⁶</td>
<td>Intravenous</td>
<td>6</td>
<td>6 × 7–11</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>5 × 10⁶</td>
<td>Intravenous</td>
<td>4</td>
<td>3 × 10–12; 17</td>
</tr>
<tr>
<td>Leucocytes</td>
<td>1 × 10⁶</td>
<td>Intravenous</td>
<td>5</td>
<td>10, 11, 15, 26, &gt; 100</td>
</tr>
<tr>
<td>Bone marrow cells</td>
<td>10–15 × 10⁶</td>
<td>Intradermal</td>
<td>5</td>
<td>3 × 7, 15, &gt; 74</td>
</tr>
</tbody>
</table>

* The animals used for these tests were either L.S.H. hamsters bearing healthy C.B. pouch skin grafts of at least 50 days standing, or M.H.A. hosts bearing C.B. grafts of similar status and standing. In all cases the immunizing grafts were derived from the original donor strain.
† Leucocytes were obtained by resuspension of theuffy coat of blood.

In this manner the pouch skin graft soon became inflamed, there were prominent intradermal haemorrhages followed by scabbing and complete necrosis. Breakdown was usually complete within two weeks.

So far we have been unable to procure rejection of established cheek pouch homografts by intradermal injection of their hosts with suspensions of dissociated epithelial cells, prepared from sheets of epidermis which had been separated enzymically from homologous pouch skin (Billingham and Reynolds, 1952). This failure can scarcely be ascribed to a lack of isoantigenicity on the part of cheek pouch epithelium in the light of the various findings presented. Present indications are that our epithelial cell suspensions were non-viable at the time of inoculation.

The “barrier” hypothesis

All the evidence presented so far strongly suggests that the anomalously long survival of pouch skin homografts stems from
a failure to sensitize their hosts adequately. One obvious hypothesis, which might account for this, is that the connective tissue of pouch skin in some way or another impedes or prevents the escape of foreign transplantation antigens from the grafts into the regional lymphatic drainage system, so that an effective antigenic stimulus fails to impinge upon the host's regional nodes.

This hypothesis has been tested experimentally in two different ways: (1) Very shallow circular depressions, about 0.5-0.7 cm. in diameter, were cut in the centres of relatively large, healthy-looking, long-established pouch skin homo- or isografts, leaving intact a continuous layer of connective tissue of cheek pouch origin. Small, thin homografts of ear or body skin were then fitted as accurately as possible into the prepared beds (see Fig. 3). Controls were provided by determining the survival times of normal skin homografts of similar size fitted to shallow beds prepared in the skin of the chests of normal hamsters.

The majority of the skin homografts “inlaid” into established grafts of pouch skin healed-in very satisfactorily, acquired a good blood supply and regenerated normal hair crops. The survival of most of them was significantly prolonged (Table V; see also Fig. 4). Established pouch skin homografts were just as effective in providing “privileged” sites for the inlaid skin homografts as
Fig. 4. Showing healthy C.B. strain ear skin homograft inlaid into a long-established pouch skin graft on the chest of an L.S.H. strain host. The ear skin homograft is now of 50 days standing.
HAMSTER CHEEK POUCH SKIN HOMOGRAFTS

Table V

Prolongation of the lives of homografts of normal skin by transplantation to shallow beds prepared in long-established pouch skin grafts, or by insertion of a layer of cheek pouch areolar connective tissue between the skin homografts and normal, full-thickness recipient areas*

<table>
<thead>
<tr>
<th>Type of experiment</th>
<th>Survival times of normal skin homografts (days)</th>
<th>No. of hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control—direct transplantation of small ear skin homografts to full-thickness recipient areas</td>
<td>16 16</td>
<td></td>
</tr>
<tr>
<td>Transplantation of homologous skin into established pouch skin isograft</td>
<td>10 1 3 2 4</td>
<td></td>
</tr>
<tr>
<td>Transplantation of homologous skin into established pouch skin homograft</td>
<td>6 2 4</td>
<td></td>
</tr>
<tr>
<td>Insertion of layer of viable homologous pouch skin areolar connective tissue between ear skin homograft and its bed</td>
<td>13 4 1 3 5</td>
<td></td>
</tr>
<tr>
<td>Insertion of layer of non-viable† homologous pouch skin connective tissue between ear skin homograft and its bed</td>
<td>17 9 2 1 5</td>
<td></td>
</tr>
</tbody>
</table>

* Throughout these experiments the hosts belonged to the M.H.A. strain and the donors were C.B. strain hamsters.
† The homologous areolar connective tissue was devitalized by repeated freezing and thawing.

were pouch skin isografts. However, when the central skin homografts underwent destruction so did their surrounding pouch skin homografts. (2) As a more direct test of this "barrier" hypothesis, sheets of the rather slimy areolar tissue, which lies beneath the tougher, compact connective tissue "dermis" of pouch skin, were stripped off pieces of homologous pouch skin and interposed between open-fit ear skin homografts (derived from the same donor), and freshly prepared recipient areas on the sides of hamsters’ chests (see Fig. 5). Again, this artifice prolonged the lives of the skin homografts, some of which exceeded 1 cm. in diameter (See Table V). New hairs were soon regenerated by
these grafts, which healed-in remarkably well in view of the adverse grafting method employed.

Since the layers of homologous areolar connective tissue employed were by no means acellular, it seemed important to determine whether their viability was essential for them to "protect" the overlays of homologous normal skin. Accordingly, a series of sheets of freshly excised pouch skin were repeatedly frozen and thawed under conditions deemed most likely to kill the cells—i.e., very rapid freezing to $-79^\circ$ followed by slow thawing (Billingham and Medawar, 1952). Evidence of the effectiveness of this treatment was provided by the failure of grafts of pouch skin or normal skin, which had been frozen and thawed three times, to give evidence of survival on transplantation to isologous hamsters. When sheets of areolar connective tissue from "devitalized" homologous pouch skin were interposed between ear skin homografts and their beds a considerable degree of prolongation of their survival again resulted.

As might be anticipated, healthy-looking, long-established central inlay skin homografts in established pouch grafts, or skin homografts overlying sheets of areolar connective tissue, were fully susceptible to states of sensitization elicited in their hosts by proxy (e.g. by intravenous injection of homologous leucocytes),

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**Fig. 5.** Showing the method of grafting homologous skin upon a layer of areolar connective tissue obtained from cheek pouch skin.
suggesting that the previous exemption of such grafts from rejection may have turned upon the absence of an adequate afferent pathway for the immunological reflex mechanism.

Various attempts have been made to bring about the destruction of long-established homografts of pouch skin by subjecting them to treatments considered likely to promote or enhance the escape of transplantation antigens from them. These included topical application of irritants, such as turpentine/acetone mixture, and croton oil, every third day over a period of several weeks. Although both agents seemed to increase the blood flow through the grafts and considerably increased the mitotic activity of their epidermis, they were completely ineffective in promoting rejection. The weekly injection of 0.25 ml. of a physiological saline solution containing 10 mg. hyaluronidase/ml. (1 mg. = 691 U.S.P. units) via a No. 30-gauge needle very superficially into the connective tissue of established pouch skin homografts, where it produced transient blebs, was also found to be ineffective. Surgical trauma too was innocuous, as evidenced by the continued survival of the injected grafts just described, and the fact that many established pouch skin homografts survived the considerable traumatization accompanying the central inlay of skin homografts.

It is very difficult to explain the wide range of survival times of pouch skin homografts displayed in each of the three donor/recipient strain combinations tested. How is it that whereas some grafts were rejected just as quickly as homografts of normal skin others survived indefinitely? Genetic variation within the strains can be excluded as a contributory factor since each strain is iso-histogenic and reacts with complete uniformity to homografts of ordinary skin. Two other possibilities are:

(1) When cheek pouch skin homografts are transplanted they carry over a variable number of donor strain leucocytes in the small amount of blood remaining in their vessels. Sometimes a sufficient dosage of these cells may survive the transient ischaemia
occasioned by grafting, get into the hosts’ vessels or tissues, and elicit an effective level of sensitization. The finding that relatively small numbers of homologous leucocytes can sensitize hosts in respect of established pouch skin homografts adds some plausibility to this hypothesis. However, no evidence was forthcoming in its favour. The fate of cheek pouch skin homografts prepared from thoroughly exsanguinated donors was found not to differ from that of similar homografts from unbled donors.

(2) When trimming the raw surfaces of pouch skin grafts to facilitate their prompt healing, lesions may sometimes be inflicted in the postulated connective tissue barrier, so that the host does get stimulated antigenically, with consequent destruction of the graft. Although it cannot be refuted, this explanation, like the barrier hypothesis itself, is not without serious shortcomings. For example, if pouch skin homografts are deliberately transplanted as very “open fits”, so that their margins are separated from host skin by an annulus of raw bed several millimetres wide, the granulation tissue that builds up becomes resurfaced, in part, by outgrowth of homologous cheek pouch epithelium which is in direct contact with host mesenchymal tissue. Although resurfaced granulation tissue is a transient structure, more or less obliterated by wound contracture, one might reasonably have anticipated, on the barrier hypothesis, that the host would have been sensitized since homografts of pure epidermis prepared from ordinary skin are antigenically effective (Billingham and Sparrow, 1954). However, the long-term survival of some pouch skin homografts of this type indicates that this does not always occur.

Influence of cortisone on fate of pouch skin homografts

Since the homograft reaction in hamsters is very susceptible to the action of cortisone (Billingham and Hildemann, 1958), which according to Scothorne (1956) acts principally by reducing the power of skin homografts to elicit an immune response, through
delaying the lymphatic vascularization among other things, an attempt was made to increase the proportion of animals which would accept pouch skin homografts for very long periods with the aid of this agent. Hosts of pouch skin homografts were given 3 mg. of cortisone acetate subcutaneously at the time of operation, followed by 1 mg. every 3rd day for three weeks. Although this treatment was very effective in increasing the proportion of animals that bore surviving grafts on the 30th postoperative day, it failed to increase the proportion of long-term acceptors of these homografts.

Discussion and conclusions

The evidence presented concerning the anomalously long survival of pouch skin homografts in hamsters is consistent with the view that their exemption from the fate of skin, or other solid tissue homografts that establish vascular connexions with their host, stems from a failure of effective amounts of their antigens to gain access to the hosts' seats of immunological response—principally the regional nodes (Mitchison, 1954; Billingham, Brent and Medawar, 1954). Various lines of evidence suggest that it is at the level of the areolar connective tissue component of these grafts that the physiological impediment must be sought. This tissue does not interfere in any way with the acquisition by the grafts of a rich blood supply, or prevent a normal skin graft from getting one when a layer of areolar tissue—living or "devitalized"—is interposed between it and a richly vascular bed. One possibility, currently being investigated, is that the majority of pouch skin grafts fail to develop an adequate lymphatic drainage for their antigens to be able to stimulate the hosts. However, it must be borne in mind that, in the case of orthotopic homografts of normal skin, immunization of the host can apparently take place in the absence of an intrinsic lymphatic drainage of the graft, the antigens of the latter entering the host lymphatics in the graft bed (see
McGregor and Conway, 1956; Scothorne, 1958; McKhann and Berrian, 1959).

We must now consider to what extent the peculiar immunological properties of cheek pouch skin homografts, transplanted to the walls of hamsters' chests, are related to the immunologically privileged nature of the site afforded to grafts of foreign origin by the intact pouches. The success of central inlays of homografts of normal skin in established pouch skin grafts on the chest suggests that a common mechanism may be involved. This belief is strengthened by our finding that survival of similar inlay grafts of rabbit skin heterografts is greatly prolonged in cortisone-treated hamsters as compared with that of orthotopic skin heterografts in similarly treated hosts. Furthermore, it has been found that a DBA mouse strain lymphosarcoma grows more rapidly when injected into established cheek pouch isografts on the chests of cortisone-treated hamsters than when injected intradermally. However, Shepro, Eidelhoch and Patt's (1960) recent demonstration that grafts of malignant melanomata, of both homologous and human origin, implanted into hamsters' cheek pouches soon provoke changes indicative of an immunological response, in the cervical nodes (which must therefore be provisionally regarded as the regional nodes of the cheek pouches), and to a lesser extent in the contralateral nodes and spleen, may indicate that a more complex or different mechanism underlies the privileged status of the intact pouch as a graft site.

Phenomena which may possibly be cognate with the prolonged survival of pouch skin homografts on hamsters' chests include: (1) the indefinite survival of a significant proportion of early foetal skin homografts transplanted orthotopically to adult rabbits reported by Toolan (1958). In discussing the exemption of these grafts from rejection this worker attached considerable significance to the incomplete maturation of the formed elements of their connective tissue ground substance. Although we have been unable to confirm Dr. Toolan's findings in rabbits, in each
of the three hamster strain combinations employed for the present study it has also been found that a significant proportion of skin homografts from near term, or early postnatal donors long outlive skin homografts from adult donors. An analysis of the mechanism involved is in progress. Preliminary results indicate that the hosts behave as if they have failed to receive an adequate immunological stimulus from the healthy-looking grafts. (2) Merwin and Hill’s (1954) observation that the survival of very small homografts of thyroid or Harderian gland tissue implanted subcutaneously into mice was greatly, if not indefinitely, prolonged if their initial vascularization was delayed for a few weeks. Nevertheless, vascularized homografts manifesting prolongation of survival remained fully susceptible to resistance elicited in the hosts by subsequent grafts of similar genetic origin.

With the exception of hamster pouch skin, the only other example of an immunologically privileged tissue is cartilage. The long-term survival of cartilage homografts is now well-documented (see Craigmyle, 1958; Gibson, Davis and Curran, 1958) and seems to depend principally upon distinctive physicochemical properties of the matrix in which the chondrocytes reside. Besides underlying the weak isoantigenicity of cartilage homografts, this matrix affords the cells complete protection even in specifically immunized hosts (Craigmyle, 1960). The situation here is comparable with that of Woodruff’s (1957) orthotopic split-thickness skin homografts on rats, separated from their hosts by a cell-impermeable Millipore membrane; and with that of homologous cells enclosed in Millipore chambers inserted into peritoneal cavities (Weaver, Algire and Prehn, 1955). In none of these circumstances were the grafted cells or tissues antigenically effective, nor could a state of pre-existing sensitization on the part of the host be brought to bear upon the potentially susceptible target cells so long as the entry of host cells was prevented.

The results of heterotopic grafting tests have shown that cornea is not an immunologically privileged tissue, though the intact
cornea is certainly an immunologically privileged site, so long as it remains unvascularized (Maumenee, 1951; Billingham and Boswell, 1953).

The fact that healthy-looking pouch skin homografts of well beyond 100 days' standing showed no evidence of loss of susceptibility to subsequent sensitization of their hosts with normal homologous skin or leucocytes lends no support to a hypothesis advanced by Woodruff and Woodruff (1950) (see also Woodruff, 1959), according to which homografts once established might eventually pass a "critical period", becoming capable of surviving an immunological resistance on the part of their host.

So far, attempts to discover other connective tissues having biological properties resembling those of the connective tissue of the hamster's cheek pouch have been unsuccessful. Nevertheless, one cannot entirely dismiss the possibility that principles similar to that responsible for the anomalous survival of pouch skin homografts in this species may be important in some naturally occurring situations in which animals are chronically confronted with potential antigens against which they do not normally react. These include the potential auto-antigenicity of certain ingredients of some organs of the body such as the adrenal, thyroid, testis, and the brain, and the potential isoantigenicity of the trophoblast which establishes such intimate contact with the maternal tissue in the placenta.

**Summary**

In the course of a study of the basis of the immunologically privileged environment afforded to tissue grafts of foreign origin by the cheek pouches of Syrian hamsters, an investigation has been made of the fate of homografts of cheek pouch skin transplanted to recipient areas prepared in normal skin. It has been found that the majority of these grafts long outlive homografts of normal skin, and some survive indefinitely. Nevertheless, well-established, healthy pouch skin homografts have been found to be
fully susceptible to a state of sensitization elicited in their hosts by homografts of normal skin or leucocytes.

Various lines of evidence have been obtained which suggest that the almost unique behaviour of pouch skin as an immunologically privileged tissue, and possibly the behaviour of the intact pouch wall as an immunologically privileged site, derive from properties of its connective tissue. In the case of pouch skin homografts this appears to prevent or impede the escape of transplantation antigens.

Related phenomena may include the long survival of foetal skin homografts in rabbits and hamsters.

The principles underlying the various findings reported may also apply as naturally occurring ancillary protective mechanisms against the risks of autosensitization in respect of potentially auto-antigenic body components, and maternal sensitization against foreign transplantation antigens in the foetal trophoblast.

Acknowledgements

The invaluable technical assistance of Miss Barbara Hodge and Mr. George H. Sawchuck is gratefully acknowledged.

REFERENCES


DISCUSSION

Woodruff: We (Woodruff, M. F. A. and Woodruff, H. G. [1950]. Phil. Trans. B. **234**, 559) were interested some years ago in analysing the curious properties of another privileged site, the anterior chamber of the eye, and they differ very sharply from those of the hamster cheek pouch. A graft in the anterior chamber will not take in a pre-sensitized animal. We were inclined to jump to the same conclusion that you have properly drawn—but it didn’t apply in our case because we showed subsequently that a graft in the anterior chamber will sen-
sitize to grafts elsewhere. The question of why anterior chamber grafts are privileged thus became a little harder to answer. The explanation we suggested is that they (a) produce sensitization slowly and (b) become more difficult to dislodge as time goes on, and that their fate turns on the balance of these processes. If the graft acquires a relatively high degree of invulnerability before it has produced a high degree of immunity, then it survives. This is very different from your results. The difference may be due to the fact that the anterior chamber lacks lymphatic connexions, whereas the cheek pouch presumably has connexions with the lymph nodes of the neck.

While we are on this subject may I add that some people tend to write about regional lymph nodes as if they were immutably the lymph nodes of a particular site. Anybody who is concerned with the surgery of cancer knows that this is not true, for if one lymphatic channel gets blocked in some way, others open up and may carry metastases to nodes which from the point of view of normal anatomy would not be regarded as regional.

One question—you said you could bring about rejection of your grafts by adoptive immunization at any stage. We found that a thyroid graft which had been in the anterior chamber for six months was not disturbed by anything we then tried. More powerful weapons have since been developed however and it might be worth trying again. In your experiments what in fact was the longest interval between grafting and adoptive immunization?

Billingham: In our experiments with long-established cheek pouch skin homografts no evidence has been forthcoming that they become progressively less vulnerable to a state of sensitivity elicited in their hosts by proxy. For example, a few million homologous leucocytes injected into hamsters bearing cheek pouch homografts of 200 days' standing will procure the rejection of these grafts within a week or two.

The finding that as few as 1 million leucocytes injected intravenously will bring about destruction of these grafts has some bearing on the question of whether the antigens in a homograft ever escape or are released into its blood vessels and sensitize the animal that way. To try and investigate this possibility a little further, we took some hamsters bearing long-established cheek pouch skin homografts and injected about 20 million homologous bone marrow cells, from the same donor...
strain, as superficially as possible into the mesenchymal tissue of these grafts. In some of the subjects this did result in prompt graft rejection, but not in all of them. However, if instead of injecting the marrow cell suspensions into the cheek pouch homografts they were injected intradermally on the opposite side of the body, the pouch skin grafts were promptly and consistently rejected. This suggests that the marrow cells do not invariably escape from their prison in the connective tissue of the heterotopic pouch skin grafts.

Medawar: It is possible that the buffy-coat leucocytes are slightly damaged somehow or other in the course of preparation, and this may make them sensitive through the intravenous route. Did you try whole blood?

Billingham: Yes. Fresh heparinized whole blood is highly effective. However, this finding left us with the question—were the red cells responsible for the graft rejections? That is why we used the purified buffy-coat leucocyte suspensions.

Brent: If you remove the epithelium of the cheek pouch by trypsinization and graft it to the chest of the hamster, what happens?

Billingham: We have tried repeatedly to separate the epithelium from the cheek pouch skin by the standard trypsin method, but it is not a favourable tissue for this procedure, and we have been unable to obtain suitable sheets of pouch epidermis for grafting experiments.

Brent: You said that you destroyed long-established skin grafts by injecting antigenic matter by other routes. But have you destroyed them by injecting presensitized cells, i.e. cells sensitized against the graft?

Billingham: No, we haven’t tried that.

Brent: It seems to me that this would be well worth trying. Quite apart from the interest that the result would carry in its own right, this would provide a fascinating opportunity to study Batchelor’s and Gorer’s phenomenon of the synergistic action of circulating serum antibody and cell-bound antibody attached to sensitized leucocytes. One could do beautiful quantitative experiments in this system, in which you have unresponsiveness without getting the host all cluttered up with donor cells.

Billingham: This is a very good suggestion. So far we have concentrated on documenting this phenomenon because we were doubtful whether anyone would believe it unless we could repeat it with three
DISCUSSION

It certainly occurred to us at an early stage that a battery of hamsters bearing long-established cheek pouch skin homografts might provide the basis of a simple all-or-none test for revealing the presence of transplantation antigens in extracts of tissues prepared from the donor strain of the pouch skin grafts.

Voisin: There is another membranous tissue which is extremely interesting in this respect: dura mater. We started to study it with Dr. Klement, a Russian surgeon who came to work with us. Unfortunately the experiments were not completed. All I can tell is the general trend of the results: in rabbits dura mater seemed to be unable to sensitize the homologous host to a further skin graft from the same donor. Orthotopic homografts of dura mater were able to remain for long periods of time and they did not sensitize against subsequent skin grafts from the same donors. This tissue seems to behave somewhat like the pouch skin of the hamster.

Billingham: As soon as evidence was obtained suggesting that the phenomenon we were studying might depend upon peculiar properties of the connective tissue of the cheek pouch skin we started to look for evidence of other types of skin that might have similar properties. Histologically human foreskin bears some resemblance to cheek pouch skin and there has been at least one claim that homografts of foreskin from newborn infants lived for a surprisingly long time. Critical re-evaluation of this possibility might be worth while. Since the thin skin of the scrotum in the rat also bears some resemblance to cheek pouch skin, we tested the fate of homografts of this tissue but found them to be rejected in the usual manner.

Medawar: Does amnion behave in the same way as pouch tissue?

Silvers: We have tried a few experiments with amnion but unfortunately we haven’t had any success. However, this may be due to lesions in the amnion since it is extremely difficult to prepare sheets of this tissue. It certainly deserves further investigation.

Michie: Can you heterograft hamster “slime” (areolar tissue) and then use it for a matrix on which to do a homograft?

Silvers: We have transplanted hamster cheek pouch to rats, and it is not accepted.

Feldman: I was very interested in your observations of the skin of the hamster cheek pouch. The only other example of which I am aware of
cell populations which are themselves not immunogenic and yet are susceptible to the homograft reaction, is some tumour sub-lines which we have established experimentally from a strain-specific sarcoma in C57BL mice. These tumour sub-lines have acquired homotransplantability: they cannot immunize the host, but they are extremely susceptible to an immune response which is formed by the original strain-specific tumour line or by other tissues of the same isoantigenic constitution. However, in our case I am not quite sure whether one can talk about a “barrier” like the one you have suggested. I shall discuss these tumours in some detail in my paper.

Barrett: Have you made any tests to see whether the epithelial structures of the skin pouch are in themselves antigenic? I would suppose that they were, but I would think that question needed to be answered.

Billingham: Our attempts to do this have so far been unsuccessful, probably for purely technical reasons. That is why I laid great emphasis on the “central implant experiment” with normal skin, to get round that sort of objection.

Barrett: This seems to me to be of extreme importance; I would think when you hit the right technique it would be found to be antigenic.

It seems to me that when you get further with your techniques that this system offers a possibility to attack a problem that bothers us all. Are we dealing here, not only with two types of response—the cell-bound and the humoral—but are we also dealing with two different classes of antigens: one the soluble type and one the particulate type that is non-diffusible and would explain Dr. G. H. Algire’s diffusion chamber experiments and Prof. Woodruff’s experiments and perhaps some of my own results? It might be possible with your techniques to show that these subepithelial barriers will pass certain well known soluble diffusible antigens but will not pass such non-diffusible antigens as are present in these epithelia.

I might suggest that there is a biological advantage to the cheek pouch of the hamster; Nature would be less than wise if she did not provide a tissue with these special properties at a site where the animal generally carries all sorts of antigenic food.

Billingham: If coarse sawdust is placed in the bottom of hamster cages sharp splinters of wood not infrequently gain access to the cheek
pouches and penetrate their walls, where they become embedded in their connective tissue. On dissecting out pouches for grafting purposes we have frequently observed these foreign bodies. Rather surprisingly there appears to have been very little local reaction to their presence. We have never seen an inflammatory or granulomatous reaction of the type that would certainly be encountered in the normal integument of the hamster or other mammals.

Barrett: How deep can these splinters go?

Billingham: They may go down to the level of the compact fibrous connective tissue of the cheek pouch.

Woodruff: But how does it reject the bacteria which are sure to enter the tissues along with the splinter? This absence of immunological response isn’t always advantageous.

Brent: Isn’t the immunological reaction itself very often harmful; the harm need not always be done by the presence of the antigen or the bacteria per se, need it?

Woodruff: There is a tendency to look at it that way, but I think it would be going much too far to suggest that the capacity for immunological reaction is of no value for survival.

Billingham: I might add that several people with whom we have been in correspondence have told us that the skin of the hamster cheek pouch is much more resistant to carcinogens than the skin of the general integument.

Hildemann: I wonder if a direct test of Dr. Billingham’s hypothesis could not be made by pre-injecting the donor with tritiated thymidine, and assuming the mitotic cells of cheek pouch skin would readily take up the label, make the usual homograft and subsequently determine the extent of DNA-labelling of the recipient’s lymph nodes. This could provide direct evidence of whether graft cells were getting into the host.

Russell: In experiments which we have not published, using tritiated thymidine-labelled skin grafts in mice, both isogenic and allogeneic, a good deal of labelled DNA can be found soon after grafting in the regional lymph nodes near both types of grafts. There may be a little more rapid release of labelled material from allogeneic than from isogenic grafts, although we aren’t very sure of this. Nevertheless it is clear, even in the isogenic combination, that there is a lot of release of
this material so one would expect labelled cells in any case and the method may not turn out to be very helpful.

Secondly, in addition to being unclear about the physiology of the efferent end of the antibody reflex arc I admit to being equally so about the afferent. I am wondering how you visualize this afferent mechanism functioning. We have tried, with Elizabeth Sparrow, to repeat Algire’s work on the question of whether specific sensitization of animals follows grafting with homologous living cells in cell-impermeable filter chambers. We have been able to achieve good survival of homologous dissociated epidermal cells in filter chambers in the rabbit for about 21 days or so, but there is no consistent evidence that these chambers sensitize, which is a thorough confirmation of Algire (Algire, G. H., Weaver, J. M. and Prehn, R. T. [1957]. Ann. N.Y. Acad. Sci., 64, 1009). I would suspect that one could dismiss this as conclusive evidence that antigens do not normally migrate freely outside of cells by saying that the antigens under these circumstances have to traverse a difficult and abnormal path to get out of the chamber. Perhaps they just don’t make it, for a variety of reasons, but are still normally released from grafts as independent molecules. Would you admit the speculation, however, that sensitization more probably takes place by a handing on, from wandering lymphocytes, of the antigenic material contained within them, to regional lymph nodes? Or doesn’t this fit?

Billingham: I find the idea of circulating lymphoid cells getting immunologically “primed” by peripherally located antigens very attractive.

Batchelor: How far down the gastrointestinal tract does this curious property go? Is it just localized to the pouch?

Billingham: It seems to be localized to the pouch. We have yet to test the oesophagus.

Brent: Dr. Billingham has suggested that there is a physical barrier which prevents a lymphatic drainage from being established. Would it be too far-fetched to suggest the possibility that there might be a chemical barrier of some kind? Maybe there are enzymes present in the connective tissue which inactivate the antigens as they come away from the epithelial cells. One way of approaching this problem would be to extract isolated pieces of connective tissue to see whether such extracts
might influence the survival time of ordinary skin grafts transplanted to the chest of the hamster.

Billingham: A colleague, thinking along lines such as those you have outlined, has suggested that the connective tissue might selectively absorb and inactivate the antigens. What appears to be a weakness of this hypothesis is the fact that repeatedly frozen and thawed cheek pouch slime will protect overlays of normal homologous skin for a long time. On an enzyme hypothesis, such as that you have suggested, one would have anticipated the necessity for the persistence of living mesenchymal cells to maintain the protective effect.

Krohn: Are you supposing that the slimy material of the connective tissue is responsible for your effect and, if so, do you think perhaps it is hyaluronic acid material?

Billingham: When I use the adjective "slimy" it is only in a purely descriptive sense. When sectioned and studied by orthodox histological methods, cheek pouch connective tissue is seen to have an abundant and well differentiated fibre content.

Krohn: Another sort of skin which does have a lot of hyaluronic acid in it is the sexual skin of monkeys. When I tried to transplant pieces of sexual skin with an underlying layer of this thick jelly-like slimy material, the grafts did not take.

Billingham: Were those thick grafts?

Krohn: They had of necessity to be thick grafts, and the epithelium overlying the dermis is very thick as well, so they may not really have had a chance.

Lawrence: Is there much known about the normal immunological capacity of the hamster in terms of induction and manifestations of delayed allergy and serum antibody formation? Could it be that it has some central immunological deficiency, even though responding rather normally to an orthotopic skin graft?

Billingham: Dr. Hildemann is more qualified to answer this than I am.

Hildemann: Hamsters respond very vigorously to a variety of heterologous antigens. For example, the injection of a moderate dosage of rat cells into the hamster will elicit a very high titre of saline haemagglutinins. The hamster will also make precipitins against protein antigens. So I think the idea that the hamster is incompetent in any general
immunological sense is hardly defensible. Although Syrian hamsters respond vigorously to various heterologous antigens, it is peculiarly difficult to induce them to make antibodies against isoantigens; we have obtained humoral isoantibodies from hamsters, but only after prolonged immunization and then not regularly.

*Lawrence:* What is their response to bacterial antigens and do they develop delayed allergy in the way the guinea pig does?

*Hildemann:* I believe Stanley Cohen has produced typical tuberculin sensitivity in the hamster.

*Billingham:* And of course the homograft reaction in hamsters to orthotopic skin grafts is highly conventional.

Dr. Hildemann has just told us how difficult it is to demonstrate humoral isoantibodies in hamsters following immunization with homologous cells. This led me to wonder whether the hamster hasn’t provided us with a sort of natural experiment to differentiate between the rôles of cellular and humoral immunity in skin homograft rejection. If you put on a skin graft and it’s rejected quite promptly you get no evidence of any humoral antibodies at all in the hamster. Is this valid evidence in trying to settle this much vexed question?

*Medawar:* Is it absence of antibody or undetectability of antibody? One doesn’t know.

*Simonsen:* May I return to this “slime”? Could you give us more details about the treatment with hyaluronidase?

*Billingham:* We injected the enzyme into established homografts to try to promote their rejection. Weekly injection for many weeks had no effect.

*Simonsen:* But did you check to see whether the hyaluronidase actually broke down some of the mucoid substance in the pouch? Have you, for example, done metachromatic staining before or after treatment with hyaluronidase? Perhaps the mucoid substance is not proper hyaluronic acid but some other mucoid substance. The fact, as you mentioned before, that the frozen-dried pouch or slime layer would also work seems to me a very strong indication that it is a sort of chemical protection.

*Medawar:* I don’t see why. Perhaps this frozen and thawed slime simply won’t support the penetration of lymphatics.

*Eichwald:* If you are unable to penetrate the slime with hyaluronidase,
did you consider trying it mechanically? As soon as it has holes in it, rejection should occur.

Billingham: Peppering these established grafts with a fine syringe needle didn’t cause rejection.

Eichwald: From the inside or the outside?

Billingham: From the outside.

Eichwald: Of course you don’t remove the plug, and it will seal up like a valve; but if you could break it from underneath by raising the skin flap and then lacerating it, that might be effective.

Billingham: That’s worth trying.

Woodruff: Is it possible you are producing a sort of local lymphatic blockade?—that some of the material absorbed from this slime finds its way into regional lymph nodes and clogs them up?

Billingham: I couldn’t exclude that possibility on the basis of our present knowledge.
MOTHER-FOETUS IMMUNOLOGICAL RELATIONSHIP AS AN EXCEPTIONAL HOMOGRAFT MODEL

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The genetic structure of the mammalian foetus developing within the mother is different from that of the mother's tissues (with the exception of intra-inbred mating). The foetal part of the placenta and the foetus itself could therefore be regarded as a homograft. Under normal conditions, however, the mother is not immunized by the tissue antigens of the foetus. Even repeated pregnancy does not lead to transplantation immunity, and females preimmunized against the tissue antigens of the breeding male can have normal offspring (Heslop, Krohn and Sparrow, 1954; Medawar and Sparrow, 1956).

Three possible explanations of the absence of a homotransplantation reaction between mother and foetus have mainly been considered: (1) antigenic immaturity of the foetus; (2) non-specifically decreased reactivity of the pregnant female; (3) the anatomical barrier between mother and foetus.

(1) The first possibility is, however, contradicted by results demonstrating the antigenic maturity of the foetus. In the pioneer studies on tumour immunity, foetal tissue has been used many times for successful immunization against homografts of tumours. Immunity induced by injections of foetal tissue has also been demonstrated by a subsequent skin transplantation test (Billingham, Brent and Medawar, 1956; Woodruff, 1958; Chutná and Hašková, 1959). As far as transplantation antigens are concerned, basic evidence of a complete, antigenic maturity of the
foetus is provided by the demonstration that tissues of very young embryos are capable also of inducing tolerance to an adult syngeneic skin graft (Medawar, 1959; Hašek, 1960).

(2) Experimental results on the immunological reactivity of pregnant females show that females either react against homologous tissues with normal capacity (Woodruff, 1958; Hašková, 1961) or display some indications of decreased reactivity. Andersen, Monroe, and Hass (1958) observed the disappearance of the classical transplantation reaction during pregnancy; Valone (1952) found a prolonged survival of homografts in pregnant female mice; and Heslop, Krohn and Sparrow (1954) found a doubly prolonged survival of skin grafts in rabbits and assumed that the ability of the mother to tolerate the foetus might depend on the increased amount of adrenocortical hormones. On the whole, however, it seems improbable that a non-specific decrease in the immunological reactivity of the mother would be decisively involved in the compatibility of the foetus.

(3) The anatomical barrier between mother and foetus could play a rôle, especially in the insulation of the reacting part before entry of the antigen, represented either by cells or antigen in an unbound form. It could, however, also prevent the antibodies or sensitized cells from passing through. Although foetal tissues are capable of inducing transplantation immunity, after repeated pregnancies in interstrain matings immunity is not induced (Medawar and Sparrow, 1956; Hašková, 1961). It seems most probable that in contact of the foetus with the mother the induction of a marked homograft reaction does not take place. Boyd (1959) has not found any cellular reaction in or near the placenta.

The nature of the placental barrier in relation to the tissues of mother and foetus is still obscure. It is known that the cytotrophoblast and syncytiotrophoblast, whose foetal origin have been demonstrated by embryologists (Hamilton and Boyd, 1960), are in intimate contact with the maternal blood, including the
leucocytes in the intervillous spaces. Fragments of the syncytiotrophoblast are actually often released and pass into the mother’s blood and lungs (Bardawill and Toy, 1959; Boyd, 1959; Park, 1959). The question is, therefore, why is an immune reaction not induced by syncytiotrophoblast and why is the transplantation reaction of the mother not directed against it? In our laboratory we have investigated whether the placenta, a part of which is of foetal origin, is capable of inducing transplantation immunity to male-specific antigens.

Mature female mice of inbred strain A mated with males of CBA strain were used. Under sterile conditions, \( A \times CBA \, F_1 \) hybrid placentas were deprived of the remnants of foetal membranes (blood was partially removed by washing in physiological saline), weighed, cut into fragments and injected subcutaneously into normal adult (2- to 7-month-old) recipients of the male or female parent strains. The age of placentas, doses and routes of administration are given in Tables I and II. The dosage corresponded to about 2–3 placentas per recipient. Three to six days after application, skin from the back of 2-4-month-old \( A \times CBA \, F_1 \) hybrid or CBA strain mice was grafted on the recipient, following the technique of Billingham and co-workers (1954). Seven days after transplantation, grafts were fixed in 10 per cent formol, embedded and histologically examined. Transplantation immunity of the recipient was evaluated according to the state of the epithelium of the test graft. Seven days after transplantation, optimal proliferation of the epithelium was found in previously non-immunized animals: in immunized animals, the epithelium was destroyed. Parallel skin transplantations of the recipients receiving other embryonic tissues under the same conditions were positive controls and grafts placed on non-treated animals were negative controls.

After transfer of 100–200 mg. of the placentas into recipients of the female strain, the recipients did not display immunity (Table I); the survival of the epithelium of the test graft was
Table I

TRANSFER OF PLACENTAS AND EMBRYONIC TISSUES INTO RECIPIENTS OF THE FEMALE STRAIN

<table>
<thead>
<tr>
<th>Age of embryos (days)</th>
<th>Type and amount of injected tissue and route of administration</th>
<th>No. of transfers</th>
<th>Day of test transplantation after inoculation</th>
<th>Percentage survival of the epithelium of the test graft</th>
<th>Mean percentage survival of the epithelium of the test grafts</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>16–21</td>
<td>Placentas 170–200 mg. s.c.</td>
<td>13</td>
<td>3–6</td>
<td>10, 25–30, 40, 55</td>
<td>70.3</td>
<td>No immunity</td>
</tr>
<tr>
<td></td>
<td>Placentas 200 mg. i.p.</td>
<td>3</td>
<td>3–6</td>
<td>90, 8 × 100</td>
<td>0, 25, 75</td>
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<tr>
<td>16–21</td>
<td>Embryos 100 mg. i.p.</td>
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<td>Embryos 100 mg. s.c.</td>
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<tr>
<td></td>
<td>Embryos 170–190 mg. s.c.</td>
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<td>0</td>
<td>Strong immunity</td>
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<td>0, 0</td>
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<td></td>
<td>0, 50, 60, 75, 10 × 100</td>
<td>84.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
70.3 per cent. In the case of negative controls the survival was 84.6 per cent, whereas following injections of other embryonic tissues, strong transplantation immunity was found; survival of the epithelium of the test skin graft reached only 5.5 per cent on the average. After transfer of the placentas into the recipients of the male strain (Table II), the mean survival of the epithelium was 26.8 per cent, which meant a good immunity; in negative controls 95.6 per cent; and in positive ones 6.2 per cent following injections of embryonic tissues. It appeared that neither the age of placentas and embryos, nor the interval between the injection and the test graft affected the results.

It can be considered that the incapacity of the foetal part of the placenta to induce transplantation immunity might be related to the occurrence of haploid nuclei in syncytiotrophoblast containing perhaps only the mother’s set of chromosomes, as assumed by Galton (1960), or that it could be the outcome of a reduced number of transplantation antigens during a rapid development of syncytiotrophoblast or of some other type of phenotypic variation in distribution of transplantation antigens.

The antigenic inferiority of the placenta demonstrated by the absence of antigens of the mating male in the foetal part of the placenta could throw a light on why transplantation reaction does not occur at the site of contact of the tissues of foetus and mother. There are, however, cases in which the placental barrier fails. Indirect evidence for such a leakage could be provided either by the occurrence of tolerance in the offspring towards the mother or by the occurrence of immunity in the mother. The occurrence of a spontaneous transplacentally induced tolerance in the mother seems to be very rare (Billingham, Brent and Medawar, 1956; Peer, Walia and Pullen, 1960; Rogers, 1958; Owen et al., 1954; Ward, Walsh and Kooptzoff, 1957); it could, however, be artificially increased under appropriate experimental conditions (Lengerová, 1957; Nathan, Gonzales and Miller, 1960), probably by the failure of the normal function of the placenta. As far
Table II

Transfer of placentas and embryonic tissues into recipients of the male strain

<table>
<thead>
<tr>
<th>Age of embryos (days)</th>
<th>Type and amount of injected tissue and route of administration</th>
<th>No. of transfers</th>
<th>Day of test transplantation after inoculation</th>
<th>Percentage survival of the epithelium of the test graft</th>
<th>Mean percentage survival of the epithelium of the test grafts</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placentas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-21</td>
<td>100 mg. s.c.</td>
<td>1</td>
<td>3-6</td>
<td>0, 0, 0, 15-20</td>
<td>26.8</td>
<td>Immunity</td>
</tr>
<tr>
<td>16-21</td>
<td>170-200 mg. s.c.</td>
<td>7</td>
<td>3-6</td>
<td>20, 75, 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Embryos</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-21</td>
<td>100 mg. s.c.</td>
<td>6</td>
<td>3-6</td>
<td>0, 0, 0, 0, 5, 45</td>
<td>6.2</td>
<td>Strong immunity</td>
</tr>
<tr>
<td>16-21</td>
<td>150-200 mg. s.c.</td>
<td>2</td>
<td>3-6</td>
<td>0, 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>—</td>
<td></td>
<td>16</td>
<td></td>
<td>35, 15 × 100</td>
<td>95.6</td>
<td>No immunity</td>
</tr>
</tbody>
</table>
as the occurrence of immunity in the mother is concerned, cases of Rh incompatibility are currently known, but they are much rarer than the theoretical immunological presumptions would suggest. Even if transfer of cellular elements between mother and foetus does not seem to be extremely rare under normal conditions (Zipursky et al., 1959), its possible immunological consequences are not very frequent.

If the placental transfer of cellular elements were equally frequent in both directions, the immunological consequences on the part of the mother should be opposite to those on the part of the offspring, reflecting the critical rôle of the stage of immunological maturity of mother and foetus for the reaction pathway which a given antigenic stimulus can initiate. From this point of view, therefore, it is rather surprising that interstrain or intrastrain parity has been demonstrated to induce a tolerance-like state to the male-strain or male-sex antigens.

Breyere and Barrett (1960a) described the effect of previous matings of BALB/c female mice to DBA/2 males on their resistance to tumour of DBA/2 origin. The tumour grew better in these post-partum females than in virgins or females parous by BALB/c males indicating a change in the natural immunogenetic resistance in the direction of some sort of “tolerance”. Later on, these authors (1960b) extended their observations to normal tissue (skin homografts) towards which a partial tolerance resulted in females from intrastrain matings. Within certain limits graft survival seemed to depend on the number of previous pregnancies, but after the third pregnancy the maximum effect available in the given system was apparently achieved.

This second result was in accord with that of Prehn (1960) who was able to produce a fairly high percentage of isologous male skin grafts in C57BL/An female mice. The degree of tolerance was roughly proportional to the number of previous pregnancies, but a partial tolerance could also be induced by matings with sterile males. Since the basis of the male sterility
(often appearing in the author’s colony) has not been investigated, the effect of some abortive pregnancies could not be excluded.

In our laboratory, the experiments of Prehn were repeated (with a different line of C57BL mice—H–2\(^b\)) using a better defined control. This consisted of matings between normal fertile males and sterile females; female sterility was produced by irradiating virgin females with two doses of 300 r. each at an interval of 2 weeks. After disappearance of cyclic vaginal cornification one isologous ovary was transplanted subcutaneously to these sterilized animals to restore the hormonal function since otherwise the females would not have mated. Under these conditions, the effect of insemination could be dissociated from that of the pregnancy. The effect of pregnancy was studied in females caged together with males (2 ♀ + 1 ♂ in one cage) to produce as frequent litters as possible. The litters were always discarded and approximately 2 months after the last litter females were grafted with isologous male skin using the usual technique. Bandages were removed on the 10th day and daily gross examinations were made until the grafts were rejected.

Experimental results are summarized in Table III. It can be seen that three or four prior pregnancies caused a highly significant prolongation of the survival time of male skin grafts transplanted to multiparous females; 23 out of 31 grafts were rejected (with a mean survival time of 33 ± 5.7 days) whereas 8 of them still survived 50 days after transplantation. Even in females which had experienced two pregnancies only (4 animals) the male-graft survival-time was significantly prolonged (26 ± 3.4 days). There was no significant difference between this value and that of 25 male skin grafts rejected by sterile mating females; in this control group, however, 3 grafts were still in place 36 days after transplantation.

This implies that even mating itself leads to a significant prolongation of male skin survival time in the nulliparous females, comparable with the effect of two prior pregnancies. Other
Table III

**RESULTS OF GRAFTING ISOLOGOUS MALE SKIN IN FEMALE C57BL MICE**

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Number</th>
<th>Mean survival time (days) of rejected grafts ± s.e.</th>
<th>Number of surviving grafts</th>
<th>Significance of difference from:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virgins</td>
<td>25</td>
<td>15 ± 1.6</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>Sterile mating females</td>
<td>25</td>
<td>24 ± 4.9</td>
<td>3 &gt; 36 days</td>
<td>Virgins: $P \leq 0.001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 pregnancies: $P &gt; 0.05$</td>
</tr>
<tr>
<td>Females after 2 pregnancies</td>
<td>4</td>
<td>26 ± 3.4</td>
<td>none</td>
<td>Virgins: $P \leq 0.001$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sterile mating ♀: $P &gt; 0.05$</td>
</tr>
<tr>
<td>Females after 3 or 4 pregnancies</td>
<td>$28\frac{1}{3}$</td>
<td>33 ± 5.7</td>
<td>8 &gt; 50 days</td>
<td>Virgins: $P \leq 0.001$</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>2 pregnancies: $P \leq 0.001$</td>
</tr>
</tbody>
</table>
factors than insemination can scarcely be held responsible for this sort of immunological inhibition but the mechanism of its effect is to be explained. According to some speculations, immunity was expected to result sometimes from repeated inseminations rather than the opposite modification of immune response but there is no critical evidence for it so far.

The fact that repeated pregnancies decrease the reactivity of females seems to indicate that perhaps another safety mechanism exists in case of failure of the barrier function of placenta. Instead of immunity, which might be expected, there occurs in parous females an inhibition of reactivity, specific towards antigens of the breed male. It seems probable that the compatibility between mother and foetus is the outcome of more than one mechanism, among which the antigenic inferiority of the syncytiotrophoblast and its function as a semipermeable membrane could play a rôle. However, the occurrence of other immunological phenomena shows that in the relationship between mother and foetus the mechanisms inducing compatibility predominate, rather than those inducing immunity. The question remains of whether an absolute mother-foetus compatibility is required for no immunological complications of pregnancy to occur, or whether such complications might not be due to a critical threshold of incompatibility being reached.

Summary

Except in intra-inbred mating, the foetus with the foetal part of the placenta may be regarded as a homograft. The foetus from inter-inbred mating does not induce an immune reaction under natural anatomical conditions but is capable of doing so after a heterotopic transfer into individuals of the female strain; however, the foetal part of the placenta is antigenically inferior in the sense that it does not immunize individuals of the female strain under conditions in which the actual tissues of the foetus would have done so.
In further experiments, a comparison is made of the degree of “tolerance” induced towards the male-sex antigen in females after repeated pregnancies and in sterile mating females. In both cases, a prolonged survival of skin grafts from the male was found as compared with their survival in virgins.

REFERENCES


DISCUSSION

Medawar: With regard to the effect of repeated pregnancies, Dr. Sparrow and I did quite a number of experiments of this kind a few years ago (Medawar, P. B., and Sparrow, E. M. [1956]. *J. Endocr.* 14, 240) and found that the results, as I am sure Dr. Barrett would confirm, do vary considerably with the strain combination that is used. In the direction A to CBA, passage through repeated homospecific or heterospecific pregnancies does not affect the survival time of skin homografts; but in grafting the other way round, from CBA to A, then passage through repeated heterospecific pregnancies does significantly prolong the life of skin homografts. It is also worth remembering that there is also some prolongation in mice which have been through repeated homospecific pregnancies—an essential control—and also in elderly virgin female mice of the same age.

Dr. Hašek, you gave three possible explanations why the foetus might not immunize the mother. I should like to suggest a fourth: that antigens do pass from the foetus to the mother by a route which is immunologically ineffective, at least as far as sensitization is concerned. Do you think this is a possibility?

Hašek: I agree that this might be a fourth explanation. However, the intravenous route of antigen administration, which is likely to be involved in the mother-foetus relationship, can by no means be considered as a *generally* ineffective form of sensitization even though this is the case as far as intravenous administration of epidermal cells is concerned.

Medawar: Oh no, certainly not; only when the antigen is in a soluble form.

Krohn: I can certainly confirm that the placental tissue is capable of stimulating an immune response, but I have done it only in the direct transfer of CBA placental tissue to an A mouse; there the subsequent test grafts have broken down as vigorously as if the animal had been immunized with any other tissue.

I wonder whether there aren’t some other possible explanations for the difference between the response of the male and female strains in your experiments. One might conceivably resuscitate the discarded
notion that the trophoblast derives from the granulosa cells surrounding the ovum and doesn’t come from the zygote. This has been suggested and totally discarded, I think, on the grounds that there were no granulosa cells to develop and that in organ culture work you only got development of trophoblast if you had removed everything including the zona pellucida. This type of difference that you have described might be used to support the notion. There is also the suggestion that the trophoblast cells may be haploid. I believe someone has estimated the amount of DNA in trophoblast cells and found that they contain half the expected amount; I don’t know how much credence should be put in that. And there may be a difference in the behaviour of haploid cells as compared to the diploid ones.

**Barrett:** I agree, in a modified form, as to the antigenicity of the placenta itself. Our test was a little different from either Dr. Hašek’s or Prof. Krohn’s. We used the hybrid combination BALB/c × DBA, as in our first report. We gave one hybrid placenta, washed free of blood, as a subcutaneous dose to a maternal-strain recipient, and we gave the foetus at 14 days and at 12 days as a single subcutaneous dose. This of course was a haploid dose of the male antigen. Each tissue produced transplantation immunity in the maternal strain as tested by the subsequent inoculation of the specific tumour. However, the embryo induced a good strong immunity (allowing for this dosage variation); the placenta induced a significant and perfectly adequate immunity, but it was not as strong as that induced by the foetus. So here again is the slight strain variation that you mentioned, here again is the slightly different dosage—I don’t know what a placenta would weigh, but I would think it would weigh 300–400 mg.

**Hašek:** There are 3 placenta for a dose of 250 mg.

**Barrett:** Then our dosage was smaller. As to the effect of difference in strain Prof. Medawar mentioned, we have recently published (1961. *J. nat. Cancer Inst.*, 27, 409) results that bear on this. In our original report the females were all BALB/c, and they were mated to BALB/c, to DBA, and to C3H. When we tested with the DBA tumour, specific tolerance was found in only those mated to DBA. Now we have found that if we introduce the C3H tumour then, as expected, it is the animal mated to the C3H male which shows tolerance. And we are getting outside the H-2 barrier.
As to this barrier between the mother and foetus, this bothers me. Dr. K. Benirschke, of Dartmouth, who has done a great deal of work on the placenta, spoke of the human placenta as having a very tight barrier which I think he ascribed to a fibrinous material. Others have talked about other types of barriers. But we are confronted with the fact that, as Dr. Hašek says, the incidence of Rh disease is less than is to be expected statistically. We think our tolerance phenomena might be a partial explanation for this. Still the antigen does get across, and whatever channel it traverses is an effective one; the mother does become effectively immunized.

Now there is one other difficulty in my mind. Dr. H. Meier (1961. Experientia 16, 215) reported a few months ago that one can distinguish, by histochemical reaction, the erythrocyte of the mother from the erythrocyte of the hybrid. He reported that the blood of hybrid foetuses contained a mixture of hybrid and maternal erythrocytes. If this can be substantiated, whatever the placental barrier is, in this combination in the mouse whole erythrocytes can cross and form a temporary chimera in the hybrid.

Hašek: I should like to come back to Prof. Krohn’s comment. Our experiments are fully in agreement with antigenic activity of placental tissue, but this applies only to maternal antigens whereas those of the paternal strain are missing when hybrid placentas are tested. The removal of blood from placentas in our experiments was certainly not complete. As to the two alternative explanations suggested by Prof. Krohn concerning the origin of placenta: if M. Galton’s (1960. Lancet, i, 495) preliminary finding of DNA content of trophoblast cells were confirmed it would be pretty direct evidence in favour of the second, more complicated hypothesis.

Barrett: We washed the placenta until no further blood could be seen coming out into the wash water. Although there probably was some blood left on the placenta, it was certainly a quite inadequate dose to have produced the immunity that we saw in the strain combination that we used.

One further point related to what Dr. Krohn said: our system was different from his; in our case any non-paternal material injected with the placenta was of maternal origin injected into the maternal strain and therefore could not have contributed to the effect we saw. It is essential
to remember that we injected hybrid material into maternal-strain recipients.

G. Klein: If it is really true that the trophoblast does not contain antigens of paternal origin, even if the nuclei are haploid the assumption that they are of zygote origin is quite untenable, because there is just no cytogenetical mechanism by which the maternal genes would segregate out from the zygote in their entirety at the time the trophoblast is formed. But I am wondering whether the negative result of the immunization experiment really does exclude the presence of paternal antigens, and whether you have made any attempt to show by absorption tests or haemagglutination inhibition that the paternal antigens are really absent.

Hašek: We did not try other techniques to confirm the absence of paternal-strain antigens in the hybrid placentas. And I would agree that a cytogenetical mechanism for the recruitment of haploid cells of foetal placenta containing only the maternal set of antigens, is rather obscure.

Brent: I am still rather worried about this question of contamination of the placentas with maternal blood cells. The experience I have had with the kind of test system Dr. Hašek has used, in which one examines 6- or 7-day-old grafts for accelerated destruction, leads me to believe that it is rather difficult to make it as precise a quantitative measure as one would like, and that the difference in degree of sensitivity in a group of mice showing, say, 25 per cent survival of the graft epithelium, and that of another group showing 50 per cent survival, is very small. Couldn’t such a relatively small difference be accounted for by the presence of maternal leucocytes in Dr. Hašek’s placentas? It is, I imagine, fairly easy to wash off red cells, but not leucocytes, which would stick to the capillaries and to the cells on the trophoblast.

Woodruff: It strikes me that there are important resemblances between the barrier Dr.Billingham discussed in his paper, the barrier Dr. Hašek has just talked about, and the artificial barriers used in diffusion chamber experiments which are permeable to large molecules but not to cells.

Indeed, my interest in the immunological problem of pregnancy arose out of diffusion chamber experiments which suggested that grafts isolated from contact with host cells failed to immunize. Our Chair-
man criticized my conclusion that host cells were necessary for the transport of antigen on the ground that grafts in diffusion chambers were undernourished and might in consequence not liberate much antigen. I set out to look for a system which was not open to this criticism and found it in the mammalian foetus (Woodruff, M. F. A. [1958]. *Proc. roy. Soc. B*, 148, 68).

I should like to comment also on a brief communication in a recent *British Medical Journal* (Hackett, E. and Beech, M. [1961]. *Brit. med. J.*, 2, 1123) in which somebody has revived the old idea of trying to treat choriocarcinoma in women by trying to immunize the patient with paternal tissues. This particular trial was not very successful, but it does seem to me that the idea merits further investigation.

**Russell:** I should like to summarize briefly some experiments on the antigenicity of mouse trophoblastic tissue which Dr. Richard L. Simmons and I have been doing. I think they bear directly on the problems which Dr. Hašek has raised.

We believe that there is clear evidence that during pregnancy the mother is quite competent to deal with foreign antigens, derived from adult tissues of the same genetic origin as its foetus, when they are presented as ordinary grafts, say, skin grafts (Medawar, P. B. and Sparrow, E. [1956]. *J. Endocr.*, 14, 240; Woodruff, M. F. A. [1958]. *Proc. roy. Soc. B*, 148, 68). It is also clear that foetal tissues contain histocompatibility antigens from quite an early stage (Terasaki, P. I. [1959]. *J. Embryol. exp. Morphol.*, 7, 409), certainly as early as 12 to 14 days of gestation in the mouse and possibly earlier (Hašková, V. [1959]. In Biological Problems of Grafting, p. 95. Oxford: Blackwell). We reasoned, in view of this, that the facts might best be explained by the "barrier theory" of immunological segregation of mother and foetus.

Theoretically the requirement is for an anatomical "buffer zone" between mother and foetus which prevents effective contact between foetal antigens and the reactive cells of the mother, a buffer which must not itself release foetal antigens.

Our experiments have made use throughout of placentas from F₁ matings between CBA and A-line mice (the paternal strain being A). Placental transplants were made to adult CBA recipients. In this way any reaction against such grafts could not be ascribed to maternal tissue fragments which had been transferred with the placenta.
Grafts of placenta, taken 10½ days after mating, and placed either topically, on bare areas prepared on the thoracic wall, or intramuscularly, are promptly rejected in the presence of a dense mononuclear cell infiltrate. Later A-strain skin grafts to these recipients are rejected in accelerated fashion. The difficulty in interpreting experiments of this kind in which placenta at this age is used, however, lies in the important fact that at this stage the placenta is a composite structure made up not only of trophoblast but also of vessels, with their contained leucocytes, and a certain amount of supporting connective tissue, all of embryonic origin. The frank sensitization which we demonstrated could, then, be due to the embryonic components of the placenta at this stage.

We thus turned to earlier embryonic stages. At 7½ days the mouse conceptus is a roughly bullet-shaped structure measuring about 0·75 mm. in length. One end is made up entirely of trophoblast, the “ectoplacental cone”, since vascular ingrowth from the foetus has not yet occurred. The other end is composed of developing embryo. It is possible to separate these two portions, more or less cleanly, under the dissecting microscope and to implant each one separately. This was done, placing one portion under the renal capsule on one side and the other under the renal capsule on the contralateral side of the same recipient. In this experiment the recipients had recently been sensitized to paternal antigens by A-strain skin grafts in order to accentuate any reaction which might occur. Although the embryonic portion usually grows and continues developing quite well after transfer to an isogenic recipient (Fig. 1), it is fully destroyed by seven days after transplantation to previously sensitized CBA recipients. Nevertheless, one can often distinguish small groups of viable trophoblastic giant cells amongst the dense mononuclears of the host, where they are apparently enjoying an anomalously prolonged survival (Fig. 2). In contrast to this, ectoplacental cone grafts survive about as well whether transferred to isologous or previously sensitized homologous hosts, with very little cellular reaction on the part of the host (Fig. 3). Degeneration of these grafts finally occurs after 12 to 13 days in both homologous and isologous situations, however, as the cells appear to undergo a “nonspecific” necrosis (Fawcett, D. W. [1950]. Anat. Rec., 108, 71).

Since slight degrees of cellular reaction had been observed against
Fig. 1 (Russell). Implant of embryonic end of divided 7-day mouse conceptus beneath kidney capsule. Both donor tissue and recipient animal were (CBA x A) F₁ hybrid. This specimen was removed 11 days after implantation and shows considerable proliferation and differentiation of embryonic tissues without appreciable leucocyte response by the host. (Haematoxylin and cosin. x 42.)

Fig. 2 (Russell). Implant of embryonic end of divided 7-day (CBA x A) F₁ hybrid mouse conceptus beneath kidney capsule of CBA mouse which had recently rejected two A-strain skin grafts. The specimen was removed 10 days after implantation. It shows complete destruction of the embryonic tissues of the implant although in the upper left and lower right corners of the field surviving trophoblastic giant cells can be seen. (Haematoxylin and cosin. x 70.)
Fig. 3 (Russell). Implant of placental end of divided 7-day (CBA x A) F₁ hybrid conceptus (the ectoplacental cone) beneath kidney capsule of CBA recipient which had twice rejected A-strain skin grafts. This specimen, removed 10 days after implantation, shows vigorous proliferation of trophoblastic giant cells with virtually no attendant host reaction. (Haematoxylin and eosin. x 70.)

Fig. 4 (Russell). Growth of (CBA x A) F₁ trophoblast beneath kidney capsule of CBA recipient which had twice rejected A-strain skin grafts. This has occurred in the 11 days since the original implant, a fertilized ovum of 8 cells. Virtually no host cellular reaction has been provoked in spite of the intimate contact between these tissues. (Haematoxylin and eosin. x 42.)
some of the ectoplacental cone grafts, possibly because of contamination with embryonic fragments during the surgical division of the conceptus, it was felt that further efforts to obtain a pure preparation of trophoblast for grafting were warranted.

This was achieved by harvesting fertilized eggs (again \((A \times CBA) F_1\)) from the oviducts two days after the appearance of the copulation plug and again implanting them beneath the kidney capsule of the recipient. In many instances these tiny structures, composed of from 2 to 8 cells, promptly developed into small tumours which were made up altogether of trophoblastic giant cells. These grafts survived in excellent condition for at least 12 days while provoking no cellular reaction. The trophoblastic cells remained in intimate contact with the homologous recipient and often infiltrated renal tubular cells. No specific sensitivity of these recipients was revealed by later skin grafting with skin of donor origin. Most striking was the fact that exactly the same vigorous development and survival of trophoblast could be readily procured on grafting fertilized eggs to animals which had recently rejected donor type skin grafts, in fact even after second-set skin grafts (Fig. 4).

The trophoblast occupies an intermediary position between the maternal and foetal circulations as a zone to which never less than two or three cells contribute at any single point (see the electron microscopic studies in the rat of G. B. Wislocki and E. W. Dempsey [1955. \textit{Anat. Rec.,} 123, 33]). We have interpreted our experiments (to be reported at length elsewhere) as giving support to the idea that the trophoblast may act as an immunological buffer zone in the mouse. It is not only properly situated anatomically, but it also appears to fulfil the requirement of being less able to provoke a reaction on the part of the host than other portions of the conceptus.

\textit{Silvers:} I would like to return to the question of parity and tolerance, and present some evidence which suggests that this tolerance is distinguishable from the tolerance which results from a neonatal inoculation of cells.

C57BL females rendered tolerant of male isografts following their inoculation at birth with isologous male cells are, indeed, cellular chimeras. Thus, as Dr. D. Wilson in our department has shown, normal females following parabiosis with such tolerant females themselves become incapable of rejecting male isografts. Furthermore, the
tolerance produced by neonatal inoculation of isologous cells can consistently be abolished by inoculating each of these tolerant animals with immunologically competent cells (from the spleen and lymph nodes) derived from a single female which has rejected a male graft.

On the other hand, C57 females which accept male grafts after having six or seven litters do not “pass” this tolerance on to normal female parabionts. Furthermore, this tolerance cannot be abolished by adoptive immunization with as many as three donor equivalents of lymphoid cells. This may suggest that these animals are not chimeras although the possibility of the persistence of Y antigen at very low levels is not ruled out.

Billingham: Prof. Medawar, haven’t you been a little too disparaging about routes of immunization in considering pregnancy and other problems? I would agree that with non-cellular and non-living antigens your points are well made, but it seems to me that it is only in the rabbit that there is evidence of a peculiarity with regard to the sensitization by the intravenous route; namely that homologous leucocytes injected intravenously in rabbits do not sensitize and may cause prolongation of survival of subsequent skin homografts and likewise with epidermal cells. But as Brent, Mitchison and I found, if you inject suspensions of splenic cells intravenously into rabbits then you do get sensitization. In guinea pigs, hamsters and mice the intravenous route is a perfectly effective sensitizing route with homologous leucocytes. I am wondering, therefore, whether the intravenous route is as peculiar as you have implied.

Medawar: I think it is not only the rabbit. As I mentioned earlier, repeated intravenous transfusions of whole blood fail to sensitize human beings. However, what I said in my paper refers only to extracted antigens in a fairly soluble form. I think that point ought to be made quite clear.

Lawrence: The interference with the development of homograft sensitivity resulting from the intravenous access of tissue antigen suggests many analogies to a similar situation in relation to delayed hypersensitive states where bacterial cells serve as antigen. In each instance, intact cells are the vehicles of antigen and in each instance the intravenous route produces an effect quite different from that produced by intradermal, subcutaneous or intraperitoneal routes of administra-
DISCUSSION

For example, it has been known for many years that for the production of delayed allergy to bacteria it is mandatory that the intradermal, subcutaneous or intraperitoneal route be used. It is of special interest that the intravenous injection of bacterial cells results in the development of an immune state (serum antibody formation) but does not result in the induction of delayed allergy. This result in relation to delayed allergy would seem generally applicable, since it has been shown to hold true for the tubercle bacillus, the streptococcus and the pneumococcus. Moreover, in tuberculous patients with delayed allergy to tuberculin, it has been repeatedly observed that invasion of the blood stream by tubercle bacilli results in the reversion of the previously positive tuberculin reaction to a negative one. These data obtained in relation to delayed bacterial allergy may be interpreted in at least two ways: either the intravenous route bypasses the cell or cells of the reticuloendothelial system concerned with the production of delayed sensitivity, intradermal and subcutaneous deposits of antigen having an obligatory voyage through lymphatics and cells of the lymph nodes; or, on the basis of the abolition of delayed allergy in the presence of a specific bacteraemia, the possibility for simultaneous sensitization and desensitization exists, the latter usually in the ascendency when antigen gains access to the circulation.
IMMUNOLOGICAL tolerance to transplanted tissues was first discovered as a state acquired during foetal or early neonatal life. This circumstance, together with the prevailing idea that the faculty of immune response does not mature until some time after birth, led the early workers (Billingham, Brent and Medawar, 1956; Hašek, 1956) to propose a scheme along the following lines:

<table>
<thead>
<tr>
<th>Developmental stage of presumptively immunological tissues</th>
<th>Characteristic modality of immunological response</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) &quot;Adaptive phase&quot;</td>
<td>Tolerance</td>
</tr>
<tr>
<td>(2) &quot;Null&quot; or &quot;neutral&quot; period</td>
<td>Nil</td>
</tr>
<tr>
<td>(3) Maturity</td>
<td>Immunity</td>
</tr>
</tbody>
</table>

The underlying idea may be termed the "qualitative hypothesis", since a change in kind is presumed to overtake the immunological system during early life. The alternative, or "quantitative", hypothesis envisages only a change in degree, and places the induction of tolerance by foetal or neonatal exposure to antigen in the same category as the immunological paralysis which Felton (1949) was able to induce in adult mice.

Two lines of work in our department have recently yielded independent and complementary supports to the latter hypothesis. In the first place, Michie and Woodruff (1962) have induced a
state of specific unresponsiveness to skin homografts in adult mice and have shown that this state bears all the hallmarks of tolerance in the sense originally defined by Medawar and his colleagues. In the second place, Howard and Michie (1962) have found it possible to immunize newborn mice with preparations of the same homologous cells which in higher dose will induce tolerance.

**Tolerance in adults**

In the past year or two many workers have induced in adult mice states of specific unresponsiveness to skin homografts by massive preliminary infusion of the intended recipient with donor cells, both by parabiosis and by repeated injection procedures. These demonstrations fall short of proving the induction of immunological tolerance, by reason of the following possibilities:

(1) The host’s immunologically competent cells might be totally replaced by cells of donor origin, as commonly occurs in radiation chimeras.

(2) The host’s immunologically competent cells might be rendered entirely inert under the impact of the antigenic overload, the host’s immune functions, as in case (1) above, being taken over by cells of donor origin.

(3) The host’s immunologically competent cells might retain their functions, but exercise them in a manner protective of the graft, namely by the production of enhancing antibodies.

These objections have all been circumvented in Simonsen’s (1960) demonstration of the acquisition of specific tolerance by grafted adult lymphoid cells of parental-strain origin towards F₁ hybrid hosts into which they have been inoculated. While logically compelling, his experiments present a feature so astonishing as to justify a certain hesitation in accepting their full implications. The donor cells appear to acquire tolerance of the host tissue within 24 hours of inoculation, and yet already in this short period to have mounted and given final effect to an immune attack
sufficient to bring their host down two or three weeks later with signs of severe graft-versus-host disease. Our acceptance of Simonsen’s interpretation has therefore been tempered by a desire to seek independent confirmation in the more orthodox host-versus-graft situation, and to analyse an induced specific unresponsiveness of the host to the graft in the light of criteria of tolerance so framed as to exclude, as Simonsen has done, all the variant possibilities previously enumerated.

**Induction of unresponsiveness in adult A mice**

Adult A-strain mice were given 350 r. or 500 r. X-irradiation, followed during the next two, or three, days by the injection of from 500 million to 1,200 million spleen cells respectively taken from (A × CBA)F₁ donors. Two treatments were administered per day, inocula being divided between the intravenous and intraperitoneal routes. F₁ hybrids were used as donors in order to obviate graft-versus-host disease, which had been found in a preliminary test to constitute a frequently fatal hazard when pure-strain CBA spleen cells were used. Three or more weeks later the treated animals were grafted with (A × CBA)F₁ skin. Table I summarizes the results, a fuller account of which will appear elsewhere (Michie and Woodruff, 1962).

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>(A × CBA)F₁ spleen cells</th>
<th>Interval before skin grafting (days)</th>
<th>Proportion of grafts surviving &gt; 2 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 r.</td>
<td>Nil</td>
<td>21–35</td>
<td>0/19</td>
</tr>
<tr>
<td>500 r.</td>
<td>5–6 × 10⁸</td>
<td>21–55</td>
<td>0/3*</td>
</tr>
<tr>
<td>500 r.</td>
<td>7–8 × 10⁸</td>
<td>18–46</td>
<td>5/7</td>
</tr>
<tr>
<td>500 r.</td>
<td>12 × 10⁸</td>
<td>21</td>
<td>8/8</td>
</tr>
<tr>
<td>350 r.</td>
<td>8 × 10⁸</td>
<td>17–37</td>
<td>0/3*</td>
</tr>
</tbody>
</table>

* One graft survived 38 days.
The higher dose levels of irradiation and of donor-type spleen cells yielded a crop of graft-bearing animals of long duration for further analysis. As a first check on specificity one of them was grafted with C57BL skin. This was rejected within two weeks with no apparent effect upon the resident (A × CBA)F₁ graft.

**Analysis of chimerism in graft-bearing A mice**

The next step was to test for the continued presence and functional activity of the host’s own lymphoid cells. The discriminant

![Diagram of discriminant assays](image)

**Fig. 1.** Design of discriminant assays estimating anti-C₅₇BL activity in the spleen cells of A tolerant (A × CBA) mice (“test”) relative to that of normal A mice (“standard”). T = test; S = standard; B = blank. An uninjected control is included to check that the B-suspension has little or no effect. All injections were i.p.
spleen assay method of Simonsen and Jensen (1959) was used, with some refinements introduced to facilitate quantitative estimation. The full design is shown diagrammatically in Fig. 1 and the results are summarized in Table II. The two assays for which rough figures only are printed are those in which imperfections of design or mishaps of execution prevented precise estimation. In order to illustrate the principles used, the measurements obtained from the remaining two assays have been plotted in Fig. 2. Since an

Table II

<table>
<thead>
<tr>
<th>Ref. No. of mouse</th>
<th>Dose of A × CBA F&lt;sub&gt;1&lt;/sub&gt; cells</th>
<th>No. of days from irradiation to skin grafting</th>
<th>No. of days from skin grafting to spleen assay</th>
<th>Estimated host-type activity as percentage of normal A-strain</th>
<th>Significant difference from zero</th>
</tr>
</thead>
<tbody>
<tr>
<td>635</td>
<td>8 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>18</td>
<td>75</td>
<td>~30</td>
<td>Yes</td>
</tr>
<tr>
<td>1295</td>
<td>8 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>22</td>
<td>54</td>
<td>14</td>
<td>Yes</td>
</tr>
<tr>
<td>1306</td>
<td>1.2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>22</td>
<td>45</td>
<td>18</td>
<td>Yes</td>
</tr>
<tr>
<td>1308</td>
<td>1.2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>22</td>
<td>101</td>
<td>≥0, &lt;20</td>
<td>No</td>
</tr>
</tbody>
</table>

approximately linear relation is known to hold between the logarithm of the dose and the logarithm of the spleen index, the log relative potency of the spleen cells of the test animal (i.e. graft-bearing A mouse) to those of the standard (i.e. normal A mouse) can be read as the horizontal distance between the fitted parallel lines.

It is evident that massive replacement of host lymphoid tissue by donor tissue has occurred. The alternative view that the reduction of activity of the host component was a long-lasting sequela of irradiation was independently checked and excluded. At the same time, three of the four analyses gave estimates of host activity significantly in excess of zero, so that total replacement or inactivation of host lymphoid cells had not occurred.
Adoptive immunization

Enhancement was earlier listed as a possible interpretation of specific unresponsiveness to skin grafts induced by earlier workers in adult mice. This possibility is not formally excluded in our own case. We have, however, been able to narrow the field somewhat by testing for the presence in our graft-bearing mice of an "efferent block", by virtue of which the graft might be enabled to survive in face of a rejection reaction on the part of the host. In such a case, the graft would be unaffected by adoptive immunity conferred by injection of isologous lymphoid cells preimmunized against the skin-donor. If, however, no efferent block exists, as in the case of tolerance, the same procedure would result in rejection of the skin graft.
Three of the graft-bearing A mice were injected intraperitoneally with 60–90 million axillary lymph node cells from A mice which had recently rejected (A × CBA) skin grafts. Three other graft-bearing A mice were selected as controls. For a long time no effect was observed, so that the presumption of tolerance seemed to be in serious doubt. At the end of the sixth week, however, the grafts of all three treated animals began to show signs of disrepair, followed, in two cases, by hair loss and scarring a few days later. The third lingered on, displaying focal eczematous lesions beneath a thinning crop of hair. Unhappily for the decisiveness of the experiment, one of the three control mice began to show a similar lingering reaction not long after the onset of rejection in the three treated animals. Were it not for this circumstance we would feel reasonably sure of our diagnosis of the graft-bearing mice as tolerant animals, in the strict sense of central inhibition. As it is, while regarding this explanation as overwhelmingly likely, we recognize the need for further reinforcement of this link in our chain of evidence. The long delay preceding the first signs of adoptive breakdown can, by hindsight, be regarded as unsurprising. In contrast with the adoptive test as applied to classically tolerant animals, in which the overwhelming mass of lymphoid tissue is of host origin (Michie, Woodruff and Zeiss, 1961), our transplanted lymph node cells had to establish themselves in lymphoid masses which, as we have seen, consisted mainly of donor (A × CBA)F₁ cells. The latter might be expected to present an absorptive screen, which the adoptive cells must first overwhelm before being able fully to deploy against the grafted skin.

**Immunization of newborn mice**

If transplantation tolerance is inducible in adults by massive administration of cells carrying donor-strain antigen, it seems possible that the variable which decides, at any given age, whether
the modality of response is to be immunity or tolerance is the antigenic dosage. On this basis, referred to earlier as the "quantitative hypothesis", it should be feasible to provoke immunity in the newborn subject in place of tolerance, simply by reducing the dosage of injected cells.

In the course of unsuccessful attempts to induce tolerance in newborn A mice by injection of lethally irradiated CBA cells (Howard and Michie, 1961), a number of cases were observed where the effects of the treatment appeared to point in the "wrong" direction. On the supposition, subsequently confirmed, that the antigenicity of the inoculum had been reduced by irradiation, so that it no longer amounted to a tolerance-inducing dose, the above-mentioned observations were followed up in detail.

The rôle of dosage was investigated by inoculating newborn pure-strain mice with varying numbers of normal (non-irradiated) spleen cells taken from F₁ hybrids between the recipient strain and the strain of the intended donor. Two days later the young mice were challenged with an inoculum of donor-strain spleen cells at a dose sufficient to induce graft-versus-host disease (measured by the splenomegaly phenomenon) in their untreated litter-mates. A high neonatal dose was expected to induce tolerance to the donor strain, and so abrogate what feeble resistance the infant hosts might offer when challenged two days later. By contrast, if low doses induced immunity, this would appear as enhanced resistance to the challenging inoculum of homologous spleen cells, with a concomitant reduction in splenomegaly towards the level of unchallenged control litter-mates. In Fig. 3 an illustrative result taken from these tests has been plotted in graphic form. While the top dose indicates tolerance, as expected, signs of acquired resistance appear at lower levels, the effect being maximal at a dose of $2 \times 10^5$ cells.

Similar resistance to challenge was also induced by neonatal injection of homologous spleen cells irradiated to 1500 r., but at a much higher total cell dose (usually 20–30 million). The pheno-
menon was regularly repeatable in each of a series of 17 tests involving 3 different strain combinations. Two of the tests included an additional type of control, namely litter-mates treated at birth with isologous in place of homologous irradiated cells. As shown in Table III, no resistance to challenge was exhibited by such animals, as predicted by the hypothesis that the phenomenon is immunological in nature. Further confirmation that the active principle in the resistance-inducing inoculum could be identified with specific transplantation antigens was obtained by extensive tests in which the neonatal and the challenging inoculum offered only a weak antigenic provocation.
Table III

Spleen indices* of CBA mice killed 8 days after challenge with C57BL spleen cells following neonatal injection with isologous or homologous lethally irradiated cells

(Data from Howard and Michie, 1962)

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 2</th>
<th>Test 481E</th>
<th>Test 482E</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7 i.v. + 10^7 i.p. irradiated spleen cells</td>
<td>1.5 x 10^7 i.v. + 1.5 x 10^7 i.p. non-irradiated spleen cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL</td>
<td>C57BL</td>
<td>CBA</td>
<td>CBA</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>+</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>+</td>
<td>2.09</td>
<td></td>
</tr>
</tbody>
</table>

* Spleen index = \( \frac{\text{rel. spleen wt. of treated mouse}}{\text{rel. spleen wt. of untreated litter-mate}} \)

to the host. In this case no resistance was conferred. The overall findings are summarized in Table IV. It can be seen that whenever the initial inoculum and the challenging inoculum have an H-2 histocompatibility group in common which is lacking from the host, the resistance phenomenon shows itself. Whenever these conditions are not met, no resistance is induced. If the resistance results from specific sensitization of the newborn mice to strong histocompatibility antigens of the challenge donor, then these results would conform precisely to expectation.

In a separate series, litters subjected at birth to the presumptively immunizing procedure were left unchallenged and their spleens were harvested on day 2 for histological examination. As compared with uninjected or isologous-injected controls, these showed an approximate doubling of mitotic rate, appearance of numerous large pyroninophilic cells in the red pulp and the emergence of foci of activated reticulum cells in the Malpighian bodies. All these changes parallel closely those found in adults following strong antigenic stimulation.

Much remains to be done if the observed phenomena are fully
### Table IV

**Summary of results obtained in a variety of strain combinations, illustrating the relationship between acquired resistance and neonatal exposure to specific H-2 antigens**

<table>
<thead>
<tr>
<th>Host</th>
<th>Donor type</th>
<th>Day 0 inoculum</th>
<th>Day 2 inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H-2 group not</td>
<td>H-2 group not</td>
</tr>
<tr>
<td></td>
<td></td>
<td>possessed by host</td>
<td>possessed by host</td>
</tr>
<tr>
<td><strong>A</strong></td>
<td>(A × CBA)F₁</td>
<td>H-2²</td>
<td>CBA</td>
</tr>
<tr>
<td></td>
<td>(A × CBA)F₁</td>
<td>H-2³</td>
<td>A</td>
</tr>
<tr>
<td>(C₃H × DBA/²)F₁</td>
<td>irrad. (ST/A × C₅₇L)F₁</td>
<td>H-2⁰</td>
<td>C₅₇L</td>
</tr>
<tr>
<td>(C₃H × DBA/²)F₁</td>
<td>irrad. C₅₇L</td>
<td>H-2⁰</td>
<td>C₅₇L</td>
</tr>
<tr>
<td>A</td>
<td>irrad. CBA</td>
<td>H-2⁰</td>
<td>C₅₇BL</td>
</tr>
<tr>
<td></td>
<td>irrad. C₅₇BL</td>
<td>H-2⁰</td>
<td>C₅₇BL</td>
</tr>
<tr>
<td><strong>CBA</strong></td>
<td>irradi. CBA</td>
<td>None</td>
<td>C₅₇BL</td>
</tr>
<tr>
<td>(C₃H × C₅₇BL)F₁</td>
<td>irradi. CBA</td>
<td>None</td>
<td>C₅₇BL</td>
</tr>
<tr>
<td>(C₃H × C₅₇BL)F₁</td>
<td>irradi. (CBA × A)F₁</td>
<td>H-2⁰</td>
<td>CBA</td>
</tr>
<tr>
<td>(C₃H × C₅₇BL)F₁</td>
<td>irradi. (CBA × DBA/²)F₁</td>
<td>H-2⁰</td>
<td>CBA</td>
</tr>
</tbody>
</table>

**Resistance**
- Yes
- No
to be elucidated. In particular the methods used could profitably be checked against independent criteria of immunity, such as skin grafting. Perhaps even more urgent is a more detailed characterization of the specificity of the effect. Preliminary studies in which irradiated ASW spleen cells were injected into A newborns, followed by challenge with CBA spleen cells, have substantiated the specificity of the phenomenon. But it is too early to say whether or not a non-specific component is also involved in the reaction.

In summary, the evidence as it stands has brought to light a novel phenomenon which it is tempting, to say the least, to interpret as immunity to homologous cells induced in the newborn by exposure to histocompatibility antigens.

Discussion

Although many past attempts to detect immunity following foetal or neonatal exposure to antigen have failed, homograft immunity in the foetal sheep was demonstrated by Schinkel and Ferguson as long ago as 1953. Three more recent successes may be quoted. Uhr, Dancis and Neumann (1960) elicited delayed-type hypersensitivity in the premature human infant; Rees and Garbutt (1961) conferred resistance to *Mycobacterium tuberculosis* by vaccination of 15-17 day foetal mice; and Silverstein, Kraner and Lukes (1962) provoked the formation of non-specific and specific γ-globulins by administering BCG, salmonella and ovalbumin to 90-day foetal sheep. These latter investigations were concerned only with heterologous antigens, and it has been suggested by Billingham, Brent and Medawar (1956) that the acquisition of immunological reactivity to heterologous antigens may occur earlier in development than to homologous antigens. Yet the fact that foetal sheep, and, as we have shown, newborn mice can react also against homologous antigen renders this idea unnecessary. We should rather discuss the observation which prompted
their suggestion, namely, the greater difficulty of inducing tolerance to heterologous than to homologous tissues, in terms of dosage-relations, and seek to know whether these relations show some form of consistency irrespective of the age of the subject.

A generally consistent principle, relating the nature of the antigen to the size of the dose required to confer tolerance, does seem to emerge, even when attention is confined to antigenic differences between members of the same species. It is well known that the tolerance-inducing dose for newborn mice is directly related to the strength of the immune reaction provoked by the same antigens administered to adults—or, as we would prefer now to say, by the same antigens administered, whether to newborns or to adults, in immunizing rather than paralysing dosage. Thus, for tolerance-induction, an H-2 antigenic difference such as CBA→A requires the intravenous injection of 4–10 million homologous spleen cells (Billingham and Brent, 1959), whereas the male-specific antigen(s) requires no more than 100,000 (Lustgraaf, Fuson and Eichwald, 1960). We have been unable to find published quantitative data for non-H-2 strain-specific antigens, but circumstantial evidence suggests that the critical dose is probably below one million spleen cells.

Interpreting, as we now feel justified in doing, induced unresponsiveness to homografts in adults as based on tolerance, we can find a similar relationship in the work summarized in Table V. By comparison with the newborn, the tolerance-inducing dose has simply been stepped up by a factor of about 200 for each category of antigen.

What, then, determines the magnitude of this factor? It is clearly not the growth in absolute mass of the animal, for the adult mouse has only about 20 times the weight of the newborn. We suggest that it is rather the total number of immunologically competent cells, which proliferate disproportionately in early postnatal life. Regarding these as the target both of immunizing and of tolerance-inducing procedures, we suggest that the issue
### Table V

**Approximate dose of spleen cells for induction of tolerance in adults**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Host</th>
<th>Nature of antigenic stimulus</th>
<th>Dose of cells</th>
<th>Host irradiation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$57$BL $\delta$</td>
<td>C$57$BL $\varphi$</td>
<td>Male-specific</td>
<td>$2 \times 10^7$</td>
<td>—</td>
<td>Mariani et al. (1959)</td>
</tr>
<tr>
<td>C$57$BL $\delta$</td>
<td>(C$57$BL $\times$ CBA) $\varphi$</td>
<td>Male-specific</td>
<td>$\leq 10^8$</td>
<td>—</td>
<td>Howard (1961) and unpublished observations</td>
</tr>
<tr>
<td>BALB/c $\delta$</td>
<td>BALB/c $\varphi$</td>
<td>Male-specific</td>
<td>$1.4 \times 10^8$</td>
<td>—</td>
<td>Lustgraaf et al. (1960)</td>
</tr>
<tr>
<td>CBA</td>
<td>(C$3$H $\times$ C$57$BL)</td>
<td>Non-H-2</td>
<td>$10^8$</td>
<td>350 r.</td>
<td>Howard (unpublished observations)</td>
</tr>
<tr>
<td>CBA $\times$ A</td>
<td>A</td>
<td>H-2$^k$</td>
<td>$8 \times 10^8$</td>
<td>500 r.</td>
<td>Michie and Woodruff (1962)</td>
</tr>
<tr>
<td>C$3$H $\times$ A</td>
<td>A</td>
<td>H-2$^k$</td>
<td>$1.5 \times 10^9$</td>
<td>—</td>
<td>Shapiro et al. (1961)</td>
</tr>
</tbody>
</table>
between tolerance and immunity may be decided, for a given antigenic difference, by one variable only, regardless of the animal's age, namely, the ratio of antigen dosage to the size of the target.

In Table VI we have set out some rough quantitative comparisons between the newborn and the adult, from which we conclude that the latter's spleen contains about 200 times as many lymphocytes as that of the newborn. If we can take the spleen as representative of lymphoid tissue, and the lymphocyte as representative of the immunologically competent cells involved in homograft reactions, we find a remarkably good agreement between the predictions of our hypothesis and the observed facts. Although the closeness of fit may be fortuitous in detail, we find encouragement in the fact that two independent assessments lead to ratios of the same order of magnitude.

This simple scheme implies that the induction of tolerance could be facilitated by the use of agents which reduce the total size of the host's lymphoid cell population. An interpretation along these lines might be applicable to the effect of prior irradiation revealed by the study on adult mice reported in this paper, and also to Woodruff's (1957) observation that treatment with

<table>
<thead>
<tr>
<th>Increase in:</th>
<th>By a factor of</th>
<th>Genotype of mice</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of lymphocytes in spleen/no. of nucleated spleen cells</td>
<td>6.5</td>
<td>C3H</td>
<td>Möller (1961)</td>
</tr>
<tr>
<td>No. of spleen cells/spleen weight</td>
<td>1.0</td>
<td>(A x C57BL)</td>
<td>Howard and Michie (unpublished observations)</td>
</tr>
<tr>
<td>Spleen weight/body weight</td>
<td>1.7</td>
<td>(A x C57BL)</td>
<td>Howard and Michie (1962)</td>
</tr>
<tr>
<td>Body weight</td>
<td>20.0</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

: Calculated factor of increase in total no. of lymphocytes in spleen = 6.5 x 1.0 x 1.7 x 20.0 = 220.
cortisone extends the period during which a given dose of homologous cells will induce tolerance in nursling rats. A natural extension of this idea into clinical practice is the possible use of anti-lymphoid agents to mediate the induction of specific tolerance to transplanted organs.

REFERENCES


DISCUSSION

Hildemann: I am most impressed with the excellence of the design of these experiments, and I think there will be many future applications of this technique. In our hands, splenic enlargement has turned out to be highly variable in graft-versus-host reactions. The occurrence and extent of splenic enlargement depend upon the stage of runt disease and
therefore on the time after injection that the assay is made. I would suggest the desirability of some independent test of immunity or tolerance beyond the criterion of spleen enlargement.

A second point is that the Friend virus, which is carried in many strains of mice, has as its chief manifestation the production of splenic enlargement on transfer to neonatal animals. This might be an additional, though one hopes unnecessary, reason why one would desire an independent assay.

Michie: On the question of desirability of independent tests of immunity, we entirely agree, but mice and time are not all that plentiful. We have started practising skin grafting to baby mice.

About the variability of spleen enlargement: everything depends, first on littermate control designs, by which you can cut down the variability by a very large factor, and secondly on the nature of your built-in controls. If the design is as it should be, it is possible to estimate what the error variation really is, from whatever cause. If there are viruses around, then they will be just as likely to manifest themselves in the untreated member of a litter or, say, an isologous-injected member of a litter. When you speak of the great variability which you have experienced are you confining attention to within-litter variation assessed in young hybrids of, say 10 or 12 days old? This is the age at which we kill them and weigh their spleens.

Hildemann: Yes. We have also used littermate controls and have given careful attention to the design you have indicated.

Brent: I don’t quite see why the transfer of sensitivity with presensitized lymph node cells should necessarily rule out the hypothesis of enhancement.

Michie: The method has been used to exclude a category of unresponsiveness under the general heading of peripheral block and efferent block.

Brent: This would apply only to unsensitized, normal cells rather than to presensitized cells. The presence of antibody does not affect presensitized cells.

Michie: It is not sufficient protection?

Brent: No, it is not. Your experiment would have to be done with normal, unsensitized cells, to permit you to distinguish between these two possibilities.
Some recent findings of ours may be relevant to your own experiments, Dr. Michie. When Billingham and I originally investigated the age-dependence of tolerance we obtained a rapidly declining curve—the number of tolerant mice declining very rapidly with age, coming down to zero by about the fifth day after birth. In these experiments we used a constant number of cells, an inoculum containing 5-10 million cells. Recently Gowland and I have been repeating this kind of analysis, but using a weight-adjusted dose of cells; in other words, we increased the number of cells with increasing age, to take into account increases in body weight of the recipients. We find that we get very nearly the same kind of curve, but the whole thing is displaced by about 4 days so that a high proportion of tolerant animals is obtained when animals are injected as late as 6 days after birth. But, nevertheless, by about the 7th or 8th day the curve reaches zero and after that a single weight-adjusted inoculum simply doesn’t produce any tolerance at all. All these injections were done intravenously. Twelve-day-old mice do not become tolerant at all; in fact, some become sensitized. We have therefore tried to find out whether there is any relationship between immunological tolerance and paralysis, by injecting multiples of the weight-adjusted dose into 12-day-old mice; we find that the greater the number of cells injected into 12-day-old mice intravenously, the greater the sensitivity! (We have injected up to 300 million cells intravenously into 12-day-old mice.)

We feel that tolerance probably can’t be entirely accounted for in terms of a sort of scaled-down version of immunological unresponsiveness in adult animals. Your experiments might help to explain these results, in terms of a cellular changeover in developing animals. If some mature cells are already present in newborn mice then that might explain why tolerance production in newborn mice should be dosage-dependent, which it is, and why it should be dosage-dependent in the first few days of life, which it also is. We feel that, in order to eliminate the mature cells (this is now following the stem-cell theory of tolerance), a prolonged stimulus is required—the kind of stimulus that Shapiro, Good and their colleagues applied by repeated inoculations of adult mice with large numbers of cells. (Gowland and I are at present investigating this possibility.) But there is no doubt that one single inoculum will not work, as it does so effectively in newborn mice.
Michie: Repeated dosage, as opposed to a single shot, in addition to having a significance with respect to time in the obvious sense, is also significant with respect to the effective dosage—because the larger the number of cells in one inoculum, the greater the fraction of that you are simply pouring down the sink. Dr. Simonsen and I have done some roughly quantitative tests of this and, at any rate in quite young (nestling) mice, this is so.

Another awkward point is that whichever point of view is right, the one that you are putting forward or the one that we have hesitantly suggested, one might still expect to find that the tolerance threshold is determined by the ratio between the dosage and the total number of mature lymphocytes in the host animal. This seems to me to make your observation at 12 days with 300 million cells not easy to reconcile with either view. It should have been enough—unless you are losing a great fraction of the single-shot inoculum by wastage, so that it doesn’t turn up in the spleen and lymph nodes.

Brent: Or unless one stipulates that tolerance does depend on the elimination of mature cells, and that in order to eliminate them by contact with antigen they first need to be exquisitely sensitized. This could be achieved by repeated doses of antigen, but not by a single dose.

Loutit: I would like to ask about the experiments on the mature irradiated animals. You suggested three explanations for the specific unresponsiveness to skin grafts in these animals: replacement, inertia, and enhancement. You have shown that there is a replacement with a final population of about 5:1 (5 of the hybrid and 1 of the host); but you test for inertia not in the chimera host itself but in the transfer system. Perhaps my question should be addressed to Prof. Woodruff: is there any clinical evidence of inertia or inactivity in the animal itself? Is the patient a good thrifty animal or a weakened animal? Are there any clinical or clinico-pathological signs in the animal itself which would suggest that these cells are inert or active?

Woodruff: The patients are doing remarkably well. I should think that the capacity to reject third-party skin would exclude the possibility that the animals were immunologically inert. Unfortunately the capacity to reject C57BL skin was tested in only two animals, and one of them died, so our evidence on this point is insufficient.

Loutit: Yes, but general immunological competence could be due to
the work of one or the other population, or both populations. In fact, what evidence have we got of the work of the individual populations in the primary host? If you take them out and transfer them, you may show that population A works, but was it working in its initial environment?

Michie: If the initial environment is wrong, then the initial animal will not be able to reject the skin graft—or not in the ordinary brisk fashion. This is a very pertinent suggestion.

Medawar: I should like to bring up a point which I don’t think was fully met in Dr. Michie’s experiments. As I remember, in one of your systems, a certain number, optimally 200,000, of adult CBA × A hybrid lymphoid cells are injected into a newborn A-line mouse. Two days later, into this same A-line mouse, are injected adult CBA lymphoid cells, and the consequence of that is that there is decidedly less splenic enlargement in this mouse than there would have been if the CBA cells had been injected without the prior injection of hybrid cells. Now, forgetting about this prior injection and considering mice that have been injected for the first time at two days with adult CBA cells, we know from Simonsen’s work that these CBA cells established in the A-line mouse soon lose their competence to react against A-line antigens (in the extreme case, in one of Simonsen’s strain combinations, in 24 hours, though we know from some of Russell’s evidence that they may continue to be reactive for perhaps four or five days). Anyhow, they fairly soon lose their reactivity. Therefore, whatever effect this prior injection has upon the CBA cells must take place pretty rapidly—in two days plus whatever time it takes for the CBA cells to become unreactive. So the first point to consider here is not merely the immunizability, if such it is, of the newborn mouse, but the great rate at which it occurs.

The second and more important point is this. If, as Simonsen’s experiment shows, these CBA cells are fairly rapidly inactivated by exposure to A-line antigen, then why are they not paralysed or rendered partially tolerant by the prior inoculation of what I should regard as an extremely massive dose of cells containing A-line antigen—namely, 200,000 CBA × A hybrid cells. An alternative interpretation would be that this prior inoculation, far from sensitizing the A-line mouse, acts upon these CBA cells in such a way as to diminish their reactivity, and
that the failure of splenic enlargement is therefore due to a sort of internecine warfare between the CBA cells and the CBA × A cells in the battleground represented by the A-line host, and is not due to an effect of the original inoculum upon the A-line mouse itself.

Michie: You mean that these donor cells are thought to be tolerant to A-line antigens introduced by the original inoculum?

Medawar: I think their reactivity may be weakened, and this might account for the diminution of splenic enlargement which you subsequently observed.

Michie: This is on the basis that the A-line antigens present in the day 0 adult inoculum are in some sense stronger or more effective?

Medawar: There are a lot of them—200,000 adult cells.

Michie: To answer this I must refer to Fig. 3 of my paper, in which the dose of donor cells was varied by ten-fold dilutions. If your interpretation were right one would expect that our phenomenon would intensify as you increase the size of the original inoculum by reason of a larger quantity of introduced A-line antigen. This is not what we found. As we increase the quantity of initial inoculum above the point which we found optimal, far from the reduction of splenic activity increasing (i.e. going down on the graph), it goes back up to the baseline, and, as one might expect on the simple hypothesis of the host being made tolerant by the larger doses, actually overshoots.

Medawar: It doesn’t overshoot until you have injected 20 million cells into a newborn mouse.

Michie: My second comment is that the bulk of our assays have been based on the alternative system that I described, where, in place of living F₁ hybrid cells we use a very much larger inoculum of lethally irradiated CBA cells, in which case this particular point doesn’t arise. The basis of our interpretation in terms of an immunizing effect on the newborn, is that in those circumstances where irradiated pure-strain cells present the correct H-2 antigenic stimulus to the host, the phenomenon shows itself, and in those circumstances where there is either no antigenic difference or there is a non-H-2 difference, we don’t get the phenomenon.

Medawar: When you use the irradiated CBA cells, Dr. Michie, how many do you inject into the newborn?

Michie: A very high dosage. Our most common was 15 million
intravenously plus 15 million intraperitoneally, and in some variants it was 10 million intravenously and 10 million intraperitoneally.

Medawar: That is a very large dose. You may think I am making difficulties, but this claim is so important that it is our bounden duty to be highly critical. Isn’t it possible that when you inject 20 or 30 million irradiated cells they are simply pre-occupying all the positions that the CBA cells injected at day 2 might otherwise have occupied?

Mitchison: That is met by the isologous controls, which are equal space occupiers, and also by the case when they are homologous irradiated cells but not presenting an H-2 difference.

Medawar: I think that meets it then.

Mitchison: I would like to raise the question of concentration as opposed to cell dosage. I find it difficult to understand why dosage per host cell can matter more than the concentration of antigen in the mouse. I feel that particularly because of my own experiment on erythrocytes in chickens where, over the first eight days of life or so, very little growth takes place and therefore the concentration of antigen produced by the injection of a given mass of cells hardly changes. Yet the total mass needed to produce tolerance increases very greatly during that period.

Mitchison: You mean that the correct ratio to look for is between antigen and, say, the total number of host lymphocytes? There is a “weak” form and a “strong” form of that point of view: the “strong” form regards the immunologically mature cell as the cell which is made tolerant; the “weak” form of the same point of view includes your belief, that it is a question of how much of the donor inoculum can get through the immune “fire” of the host for long enough for a new population of cells to grow up in the presence of the antigen and the old population of immunized cells to die away. I think you referred to these two theories in a review recently as the homogeneous and the heterogeneous hypotheses. It would be nice to find critical ways of distinguishing between them. It is quite possible, though, that they are both compatible with the “weak” form of our suggestion, that ease of induction of tolerance would be inversely proportional to the strength of immune resistance put up by the host, which in turn would be correlated closely with the total lymphocyte population. But we have no intention of pressing the “strong” form.
Mitchison: Once one accepts your conclusion that newborn mice can be immunized, doesn’t this imply that a comparison between paralysis and tolerance cannot be drawn from your experiments? The question is, if you put antigen in before the animal can react, will the dosage which is needed then be very much less? So in a sense your own experiments have forced you into injecting the mice in utero if you really wish to answer the question.

Michie: This gets us into terminological troubles, as to precisely how the terms of paralysis and tolerance should be used: whether to erect a distinction by definition or to regard the distinction as a distinction in principle between two forms of central inhibition.

Mitchison: There is a distinction in principle: but if you want the antigen there from the beginning, before reactivity develops, you may have to inject the antigens into the cytoplasm of the ovum! There must be a point before reactivity is present. And the question is, if antigen is present from before that stage, will less be needed for inhibition?

Hildemann: We were not especially surprised to find that preparations of small lymphocytes from C57 mice failed to induce any runting symptoms whatsoever in A-line mice older than 24 hours of age. Neither were we surprised on injection of such preparations at dosage levels of 2 million cells from C57 to F1 hybrids to find that we got runting symptoms only in hybrids less than 48 hours of age. But when we increased the lymphocyte dosage, as Simonsen and others have done, we still failed to get runt disease in F1 animals injected after 2 days of age. What the basis for this is I don’t know, although I have a suspicion that F1 hybrid vigour might be involved—since these F1 animals gain weight at a much more rapid rate than do the parental-line animals.

With respect to your experiments, I wonder if the so-called neutral or null period really exists. It seems strange that a mouse which can readily be made tolerant up to 24 hours of age should then enter a period when it will become neither immune nor tolerant, and then at about a week of age it may become immune. Surely there must be a transitional stage between tolerance responsiveness and immune responsiveness.

Michie: Our own point of view on that is that the original null period in fact corresponds to a null zone in dosage level. There is a
maximal response in the direction of immunity and then as you increase the dosage, it turns over into its opposite and takes the form of tolerance. We expect that you could reproduce this curve, with its null zone, in animals of any age by adjusting the whole dosage scale correspondingly.

Medawar: Proportionate adjustment of dosage scales?

Michie: No. We have talked cabalistically about a figure of 200-fold which was based on the comparison between tolerance threshold in the newborn and the tolerance threshold in the adult, and a similar figure of 200-fold is reasonable for the difference between the immunizing threshold in the newborn and the immunizing threshold in the adult—if what we have shown is immunity. That second point is much more shaky but I think it is possible that you would have to shift the whole dosage scale up by a factor of the order of hundreds.

Woodruff: In speaking of dosage, though, one ought perhaps to emphasize the “effective” dose. This is illustrated by another part of the experiment in the adult animals in which we found that if the recipients were splenectomized, then the same dose of cells didn’t produce acceptance of the graft but produced immunity. The most obvious explanation is that splenectomy revives one of the sites in which the injected cells normally settle, and in consequence many of them perish for want of a home.

Michie: I have a comment in connexion with the point made by Dr. Hildemann, about the effect of changing the dosage on the response to injection of parent-strain cells into F₁ hybrids. This is using intraperitoneal injection and taking spleen enlargement as the index of the reaction—which is an index of an early stage in the reaction. Then if you adjust dosage, that is the number of cells, to the body weight of the animal you get the same response right the way from birth up to adult life. I think that may be relevant here. The disproportion comes in when we are considering tolerance induction and not the capacity of the inoculum to settle in the business parts of the animal and to produce a graft-versus-host reaction.

Brent: Gowland and I would agree with Hildemann that the neutral period is probably a myth. The experiments which I mentioned earlier in this discussion indicate that an animal, which might have been in the neutral period as far as a dose of five million cells is concerned, is
by no means “neutral” when it is injected with a larger number of cells. In this critical first postnatal week tolerance induction is therefore entirely dose-responsive. The work of Silvers and Billingham, for example, has shown that it depends also on the strength of the antigen, which in itself may depend on the genetic relationship between the donor and the recipient; thus for the sex-linked antigen the period in which tolerance can be induced after birth is very much longer than for the H-2 or the H-3 antigens.
IMMUNOGENETICS OF TUMOURS GROWN IN RADIATION CHIMERAS

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Tumour transplantation in radiation chimeras has been studied from three distinct viewpoints: (1) to test the immunological competence of radiation chimeras, and the cellular origin of the immune response; (2) to study certain aspects of the tumour-host relationship, particularly host factors determining the formation of tumour metastasis; and (3) to study changes in the immunogenetic properties of tumour cell populations. The present discussion will deal with the problem of tumour metastasis, and with the immunogenetic properties of tumour cells, as revealed by transplantations in radiation chimeras.

Table I

THE FORMATION OF A HOMOGRAGT REACTION AGAINST TUMOUR TRANSPLANTS IN ISLOGOUS RADIATION CHIMERAS

<table>
<thead>
<tr>
<th>Tumours grafted</th>
<th>Radiation chimeras</th>
<th>Tumour-bearing animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCIM</td>
<td>C57BL spleen cells → C57BL</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td>C57BL foetal liver cells → C57BL</td>
<td>7/7</td>
</tr>
<tr>
<td>C10</td>
<td>C57BL spleen cells → C57BL</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>C57BL foetal liver cells → C57BL</td>
<td>5/5</td>
</tr>
<tr>
<td>C3H</td>
<td>C3H spleen cells → C3H</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>C3H foetal liver cells → C3H</td>
<td>10/10</td>
</tr>
</tbody>
</table>

Table I illustrates the type of tumour chimera system used in our experiments, and the capacity of the donor cells to form transplantation immunity against the tumour homograft. We
used both a tumour of recent origin, the sarcoma C10, produced in C3H mice by 3 : 4-benzpyrene, and a long-transplanted tumour of C3H origin, the sarcoma MC1M. It is seen (Table I) that infant spleen cells from 8-day-old donors, following colonization of lethally irradiated isologous hosts, can induce transplantation immunity. The cells (spleen or foetal liver) were inoculated 24 hours following X-irradiation, and the tumours were grafted subcutaneously immediately afterwards. The rejection of homografts of both tumours by the genetically foreign spleen chimeras, and their progressive growth in the foetal liver chimeras, indicate that the immune response has been produced by the donor-type cells. The rejection of the MC1M homografts by the C57BL chimera shows, in fact, that the immune response produced by the donor cells is intense, since this tumour possesses the capacity, under certain normal conditions, to resist the homograft reaction (Feldman and Sachs, 1957).

It should, however, be pointed out that different results were obtained when isologous bone marrow chimeras were tested for their immunological reactivity (Barnes et al., 1957; Koller, Doak and Davies, 1961). Isologous BALB/c bone marrow chimeras behaved similarly to BALB/c foetal cell chimeras when challenged with a homograft of the C3H-originating sarcoma BP8: in both groups a progressive growth of the homograft was obtained when the tumour was challenged on days 0 to 25 following bone marrow transplantation. Similar results were obtained when skin was used as a test homograft (Koller, Doak and Davies, 1961). It thus appears that infant spleen cells, following repopulation, confer on the host a high degree of immunological reactivity, whereas the immunological reactivation of bone marrow chimeras is delayed.

**Tumour metastasis**

Malignancy in human sarcomas is generally associated with the progression of tumour metastasis. On the other hand, most
of the experimental mouse sarcomas, particularly those induced by polycyclic hydrocarbons, grow progressively at the site of the primary tumour formation, or at the site of transplantation, but only a few produce metastasis. Hence, attempts to study experimentally factors involved in the formation of tumour metastasis in mice have been very limited. Experiments on transplantation of tumours in various combinations of radiation chimeras seem to have furnished a new system for the experimental analysis of this phenomenon. All sarcomas used in our experiments were of the "non-metastatic" type, i.e. they did not form progressive metastasis when grafted either subcutaneously or intramuscularly in normal, non-irradiated mice. However, when these tumours were grafted to radiation chimeras, a regular appearance of metastasis was observed in the inguinal, brachial, and axillary lymph nodes. The results, summarized in Tables II and III, show that sarcoma C10, of C3H origin, developed

Table II

TUMOUR METASTASIS PRODUCED IN RADIATION CHIMERAS BY SARCOMAS C10 AND MC1M

<table>
<thead>
<tr>
<th>Expt. group no.</th>
<th>Tumour</th>
<th>Host</th>
<th>Growth of primary graft</th>
<th>Growth of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C10</td>
<td>Foetal C3H → C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>C10</td>
<td>Spleen C3H → C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>C10</td>
<td>Foetal C57BL → C57BL</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>C10</td>
<td>Spleen C57BL → C57BL</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>C10</td>
<td>from expt. 2 Normal C3H</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>C10</td>
<td>metastasis from expt. 2 Normal C3H</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>MC1M</td>
<td>Foetal C57BL → C57BL</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>MC1M</td>
<td>Spleen C57BL → C57BL</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>MC1M</td>
<td>from expt. 7 Normal C3H</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>MC1M</td>
<td>metastasis from expt. 7 Normal C3H</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Expt. group no.</td>
<td>Tumour</td>
<td>Host</td>
<td>Growth of primary graft</td>
<td>Growth of metastasis</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>-------------------------------------------</td>
<td>-------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1</td>
<td>SBL1</td>
<td>Foetal C3H→F1(C3H × C57BL)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>SBL1</td>
<td>Foetal C57BL→F1(C3H × C57BL)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>SBL1</td>
<td>Foetal C57BL→C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>SBL1</td>
<td>Foetal C3H→C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>SBL1</td>
<td>Spleen C3H→C3H</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>SBL1</td>
<td>from expt. 4</td>
<td>Normal C57BL</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>SBL1</td>
<td>from expt. 4</td>
<td>Normal C3H</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>SBL1</td>
<td>metastasis</td>
<td>Normal C57BL</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>SBL1</td>
<td>from expt. 4</td>
<td>Normal C3H</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>S2</td>
<td>Foetal C3H→C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>S2</td>
<td>Spleen C3H→C3H</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>S2 from</td>
<td>Normal Swiss</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

Table III

Tumour metastasis produced in radiation chimeras by sarcoma SBL1 and mammary adenocarcinoma S2
metastasis only in an isologous system, i.e. when the tumour, the inoculated haemopoietic cells, and the irradiated host, were of the same isoantigenic constitution (Table II, groups 1 and 2). No metastasis appeared when sarcoma C10 was grafted in homologous chimeric hosts, i.e. in irradiated C57BL mice protected by C57BL cells (Table II, groups 3 and 4). It should be noted that in homologous hosts produced by the injection of foetal C57BL cells into C57BL mice, the primary subcutaneous transplant of C10 grew progressively (Tables I and II), yet no metastasis appeared. On the other hand, sarcoma MCIM showed a regular progression of metastasis in homologous chimeric hosts produced by foetal C57BL cells inoculated into C57BL mice (Table II, group 7). Homografts of sarcoma SBL1, produced in C57BL animals by 3:4-benzpyrene, showed progressive growth of the original transplant in the two groups of homologous chimeric hosts, i.e. in irradiated C3H mice protected by C3H spleen cells, and in irradiated C3H mice protected by foetal C3H cells (Table III). Yet metastasis developed only in the homologous animals protected by foetal cells (Table III, group 4). A spontaneous mammary adenocarcinoma, S2, which developed in a Swiss mouse in our colony, did show the formation of metastasis in both types of homologous chimeras: in irradiated C3H protected by C3H foetal cells, and in irradiated C3H protected by C3H spleen cells (Table III, groups 10 and 11). This tumour, unlike the previous ones, gave successful homografts when transplanted subcutaneously to normal, non-irradiated C3H hosts. It thus appears that in addition to the effects of total body irradiation, which promoted the formation of metastasis in isologous systems, certain immunological conditions, manifested in homologous systems, are among the determining factors in a host-tumour relationship which control the development of metastasis. Lymph node metastasis seems to be more susceptible to the immune reactions in incompatible chimeras than the primary transplant. Thus, a C10 homograft in foetal cell chimeras grew progressively, yet no
metastasis developed. The SBL1 homograft did grow in spleen cell chimeras, but metastasis appeared only in foetal-cell treated animals, where the immune response is reduced. Whether the promotion of metastasis in isologous systems is also due to some kind of reduced host resistance, is still an open question.

The formation of metastasis in X-irradiated isologous systems could theoretically be attributed to either of the following processes: (1) that the destruction of the lymphatic tissue by X-irradiation furnishes favourable physiological conditions for tumour cell migration, which is restricted in animals with normal lymphatic tissue; or (2) that cell migration from the original implant, via the lymphatics, takes place even in normal animals, but that the progressive growth of the tumour cells within the normal lymph nodes is suppressed. The X-irradiation accordingly alleviates a suppressing effect exerted by the organized lymph nodes. To test these two assumptions, tumours were grafted subcutaneously into isologous C57BL animals, the lymph nodes were extirpated at various time intervals following grafting, and nodal cell suspensions from individual hosts were injected intramuscularly into other isologous animals. It was found that re-inoculation of lymph node cell suspensions from each of the primary hosts 25 days following subcutaneous transplantation of SBL1, resulted in tumour growths in all secondary hosts. Hence, metastatic migration takes place even in non-irradiated normal animals, but within the organized normal lymph nodes the progressive growth of such tumour emboli is suppressed. This suppressive effect is abolished by X-irradiation.

The formation of metastasis in radiation chimeras was also noted by Barnes et al. (1957). In non-irradiated animals, cortisone was found to activate the formation of metastasis in otherwise non-metastatic tumours (Agosin et al., 1952; Baserga and Shubik, 1954). Furthermore, Baserga and Shubik have shown that cortisone, like X-irradiation, has in fact elicited the progressive growth of pre-existing metastatic emboli. In view of the simi-
Immunogenetics of tumours in chimeras

Immunogenetic changes in tumour cell populations

In contrast to the results obtained by rejection of homografts of two strain-specific tumours by genetically foreign spleen cell chimeras, which are presented in Table I, transplantation of a third tumour, the sarcoma SBL1, gave different results: here, even transplants on foreign spleen chimeras (C3H→C3H) gave successful homografts. Lethal takes were obtained both in foetal-treated and in spleen-treated C3H hosts. The progressive growth of the SBL1 in immunologically reactive chimeras could be attributed either to a low susceptibility of the tumour to the immune response formed in the C3H chimera, or to changes
taking place within the tumour cell population while growing in the radiation chimera. However, while transplantation tests in normal animals showed that the SBL1 is more susceptible to the homograft reaction than the MC1M (see Feldman and Sachs, 1957), the MC1M was rejected by the “foreign” spleen cells of the chimera, whereas the SBL1 was not. This made the second assumption—the possibility of changes induced in the immuno-genetic properties of tumour cell populations—more feasible, and experiments were performed to test this.

Sarcoma SBL1 was grafted to C3H foetal-cell treated chimeras, and both the tumour of the original transplant and that of the lymph node metastasis were removed and tested for transplantability in normal homologous hosts. The results (Fig. 1) showed that following one transplant generation of SBL1 within the C3H chimeras, a change takes place in the tumour cell population, which was manifested in 20–50 per cent lethal takes of the tumours in non-irradiated secondary hosts. Further serial transfers through 3–4 transplant generations established homotransplantable tumour lines (SBLx sublines), which gave 100 per cent lethal takes in foreign hosts. The homotransplantability acquired by tumour cell populations in C3H chimeras, and then selected out in C3H normal hosts, showed no strain-specificity: the SBLx sublines established in C3H animals gave lethal takes in animals of H-2D, H-2K, H-2B and H-2S genotypes.

Such changes, manifested in an acquired homotransplantability of a strain-specific tumour, were repeatedly obtained with sarcoma SBL1. In order to obtain some insight into the mechanism of induction of the loss of strain-specificity, we attempted to test (1) the immunogenetic factors in the chimera which control or influence such changes, and (2) the properties acquired by the “changed” tumour cell population which make possible the progressive growth of the tumour in genetically foreign hosts.

Some years ago, Koprowski, Theis and Love (1956) described
another experimental system in which an induced homotransplantability of strain-specific tumours was achieved. The “inducing” environment, in their case, was embryos of foreign genetic constitution, or “foreign” newborn which had been made tolerant to the isoantigens of the tumour. A comparison of the genetic properties of the two systems points to two possible factors which might influence such changes: (1) a low immune response of the inducing environment; and (2) the prolonged growth of the tumour in a foreign antigenic background.

Fig. 1. The establishment of homotransplantable SBLx sublines, from SBL1, grafted on isologous C3H chimeras.
The first factor might exert its effect through an antigen-antibody interaction, which could result in an immunoselection of compatible cells. The second points to some interactions between cells of different antigenic constitution. Since radiation chimeras of various genetic combinations can be constructed, this system can be used to differentiate between the relative importance of these two assumed factors, i.e. between the function of the low immune pressure on the tumour cell population, and the function of the antigenic “foreignness” of the host.

A series of experiments was carried out in which SBL1 was grown for 27 days in the following chimeric animals, and then tested for acquired homotransplantability in C3H animals:

1. Irradiated C3H treated with foetal C3H cells.
2. Irradiated C3H treated with foetal C57BL cells.
3. Irradiated F1(C3H × C57BL) treated with foetal C3H cells.
4. Irradiated F1(C3H × C57BL) treated with foetal C57BL cells.

The criteria for the evaluation of the intensity of the change in each of the experimental groups, were (a) the number of lethal takes of the SBL1 in the secondary non-irradiated C3H hosts, (b) the growth period of SBL1 in the secondary C3H prior to regression (in those cases which did not give lethal takes), and (c) the maximal size attained by the tumour homograft in C3H animals.

The results (Feldman and Yaffe, 1959) showed that the most intense change takes place in C3H→C3H chimeras. The C57BL→C3H were also found to be good inducing hosts, although to a somewhat lesser degree than the C3H→C3H animals. Tumours growing in the C3H→F1(C3H × C57BL) chimeras showed a prolonged survival in the secondary normal C3H, but without lethal takes. A definite change towards homotransplantability was observed, but homotransplantable sublines were not established. The C57BL→F1(C3H × C57BL) were the weakest “inducing” hosts.
Had the immune pressure been the determining factor in the C3H→C3H animals, it could be expected that the C3H→F1(C3H × C57BL) chimera would be a more active inducing host than the C57BL→C3H, since both C3H→C3H and C3H→F1(C3H × C57BL) have been repopulated with C3H, which is the immunologically reactive element in the chimeras. And yet the C57BL→C3H induced a much more intense change in the tumour cell population. Had any direct correlation existed between the degree of the immune response which SBL1 might elicit, and the degree of loss of strain-specificity by the tumour cells, the order of potencies of the inducing hosts would have been the following:

C3H→C3H > C3H→F1 > C57BL→C3H > C57BL→F1.

However, the order of actual potencies found in the experiment described was:

C3H→C3H > C57BL→C3H > C3H→F1 > C57BL→F1.

This order represents a gradient of antigenic components within the chimera which are foreign to the SBL1 tumour: C3H components, which are highest in C3H→C3H and lowest in C57BL→F1. The C57BL antigens (isologous to the tumour), seem to exert an inhibitory effect on the loss of strain-specificity. Whether, in fact, the changes are due to interaction between tumour cells of one genetic constitution and X-irradiated cells of a different genetic constitution, is a possibility which must await further experimental clarification.

Properties of the adapted tumour

The homotransplantability acquired by the adapted tumour could be attributed to either of the following mechanisms:

(1) Acquired resistance to the isoimmune response. Tumours of this property will elicit an intense homograft reaction, which, however, they can resist (Feldman and Sachs, 1957).
(2) Loss of histocompatibility genes, and subsequently of antigens. Tumours of this property will not elicit an immune response, and will not be susceptible to an immune response produced by other tissue homografts of the same isoantigenic origin (Hauschka et al., 1956; Amos, 1956).

(3) Suppressed production of the strain-specific isoantigens. Tumours of this property will not elicit an immune response, but will remain susceptible to a homograft reaction produced by other tissue homografts of the same isoantigenic origin.

Experiments were therefore designed to test the homotransplantability of the SBLx sublines in relation to each of the three postulated mechanisms. In one representative experiment, the following groups were set up:

(1) C3H mice were challenged with two simultaneous intramuscular grafts of SBLx, one in each leg.
(2) C3H mice were challenged with two simultaneous grafts, one leg receiving the strain-specific SBL1, the other the SBLx.
(3) C3H mice were challenged with two simultaneous grafts, one leg receiving normal C57BL spleen cells, the other the SBLx.

Table IV

<table>
<thead>
<tr>
<th>Expt. group</th>
<th>Inoculated leg</th>
<th>Graft</th>
<th>Tumour growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Right</td>
<td>SBLx</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>SBLx</td>
<td>5/5</td>
</tr>
<tr>
<td>2</td>
<td>Right</td>
<td>SBL1</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>SBLx</td>
<td>0/5</td>
</tr>
<tr>
<td>3</td>
<td>Right</td>
<td>C57BL</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>spleen</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SBLx</td>
<td>0/5</td>
</tr>
</tbody>
</table>

The results (Table IV) show that the SBLx is susceptible to a homograft reaction produced by tissue grafts of its strain of origin (C57BL). Regression of the SBLx took place when the tumour was grafted simultaneously, either with the strain-specific
IMMUNOGENETICS OF TUMOURS IN CHIMERAS

subline SBL1, or with C57BL spleen cells, whereas simultaneous transplantation of two SBLx grafts resulted in lethal takes of both. Since experiments with resistant tumours showed that homografts of such tumours grow progressively when grafted simultaneously with strain-specific tumours of the same genetic origin (Yaffe and Feldman, 1959), it was thus suggested that the SBLx belongs to the third category of the homotransplantable tumours. Further experiments were carried out to test whether the antigenicity of the tumour, measured by its capacity to elicit graft rejection, is indeed low, or whether its action is merely delayed, and develops after the tumour is already established. Two groups of C3H mice were grafted subcutaneously, one with SBL1, the other with SBLx. Seven days later, the tumours were extirpated, and animals of both groups were grafted two days later with SBLx. The results showed that a first graft of SBL1 elicited a homograft reaction that prevented the growth of a second SBLx (1/15), whereas following a first SBLx graft, 10/15 of the animals showed lethal takes of the tumour. It thus appears that the SBLx is highly susceptible to the homograft reaction elicited by isoantigens of its strain of origin. Yet its own antigenicity is very low.

Mechanism of loss of strain-specificity

In most of the experimental demonstrations of conversion of strain-specific to non-specific tumours, long serial transplantations have been involved. The only report on homotransplantability acquired through transfers in genetically foreign X-irradiated animals (Krebs, Thordarson and Harbo, 1942) is one in which serial passages during 7 years were involved. It seems probable, therefore, that in all these cases a gradual selection of "compatible" cells took place. In our experimental system one transfer generation was sufficient to induce the loss of strain-specificity, which makes the assumption of immunoselection rather improbable. The conditions within the chimeras, which were found to be
compatible with the induction of immunogenetic changes, seem to have no direct correlation with the immunogenetic conditions within the chimeras. Furthermore, the final result with tumour lines susceptible to the homograft reaction, but themselves not immunogenic, can hardly be reconciled with a selective pressure involving an immune mechanism. An immunological pressure could have selected out either cells which can resist the homograft reaction, or which have lost the histocompatibility antigens, the target for the homograft reaction. SBLx cells, being susceptible to the homograft reaction, could have no selective advantage. It seems relevant to point out that, unlike our experiments on the immunizing effect against SBLx of a simultaneous graft of SBL1, the experiments of Klein and Klein (1954) on the transplantation of a mixed population of his F1-adapted tumour cells and the original strain-specific tumour cells resulted in the selection of the adapted tumour, which gave 66 per cent of lethal takes in F2 animals. However, although a process of immunoselection does not seem to play a decisive role in the mechanism of adaptation of SBLx, other processes of selection cannot be ruled out. To differentiate between a selective mechanism and a mechanism of induction of direct genetic change within the tumour cells, we are trying now (1) to test the incidence of change when lower cell doses are transplanted in radiation chimeras, and to apply the fluctuation test to results of adaptation experiments, and (2) to establish clones of the strain-specific tumours, and test the incidence of acquired homotransplantability in individual clones.

The properties acquired by the homotransplantable tumours, namely their decreased immunogenicity, associated with a high susceptibility to the homograft reaction, require further analysis. The apparent suppression of antigen production could derive either from a quantitative change, or from a qualitative change, of the original H-2 genetic complex. A quantitative change, such as a repression or switching-off in gene action of the H-2 complex, might have caused a low production of antigen, below the
immunogenic dose. A qualitative change can be visualized as a change in the antigenic structure, which could confer on the H-antigens of the SBLx cells properties of haptens. Like haptens, such antigens will be devoid of immunogenic properties, but will be susceptible to the antibody response produced by the "complete" antigens of the SBL1 or C57BL spleen cells.

It may be pointed out that changes in the immunogenetic behaviour of haemopoietic cells, following their exposure to the foreign isoantigens of the X-irradiated host, have recently been reported. Koller and Doak (1960) found that BALB/c bone marrow cells, injected into lethally irradiated C57BL, and then retransplanted into secondary and tertiary C57BL hosts, showed adaptation to their foreign hosts, manifested in an increased survival of the secondary and tertiary homologous chimeras. A similar conclusion was drawn by Ilbery (1960) and Ford, Ilbery and Winn (1961) from serial transplantation of foetal and spleen cells in homologous hosts. It appears that the duration of exposure of donor cells to the isoantigens of the irradiated host might determine the "adaptation". In our experiments we found that the tumours must grow for more than 25 days before they reveal the loss of strain-specificity. In bone marrow experiments it has been shown that a short exposure of donor cells to the foreign isoantigens of the host results in a secondary response when such cells are reinoculated to secondary irradiated homologous hosts (Feldman and Yaffe, 1958; Popp, 1961). If, however, the foreign bone marrow cells are exposed for a prolonged period (more than 30 days) and then retransplanted, an adaptation to the foreign host is observed (Popp, 1961). These processes of adaptation, unlike the changes described in tumour cells, seem to be host-specific: if the "adapted" cells are inoculated to a third, unrelated host, they produce the usual secondary disease (Popp, personal communication). The two processes of change, in bone marrow and tumour cells, seem to be of a different nature. One is a specific suppression of antigen production, while the other is a
specific suppression of antibody production. The latter might be a result of immunological paralysis, due to prolonged exposure to a high dose of isoantigens of the hosts. However, unlike other phenomena of paralysis, this one is preceded by a stage of immunity (following a shorter exposure time). Thus, the two mechanisms of "adaptation", of bone marrow and of tumour cells in radiation chimeras, are still unclear. They both seem to reflect results of interactions between cells of different antigenic constitution.

REFERENCES

DISCUSSION

Medawar: Do these transformed tumours provoke the formation of humoral antibodies?

Feldman: These tumours have a peculiar behaviour in relation to humoral antibodies. Unlike other tumours with which we have been working in C3H mice, these tumours of the SBL1 and SBLx sublines do not elicit isohaemagglutinins when grafted in foreign strains. However, isohaemagglutinins which are formed by homografts of normal tissues of the same genetic constitution (C57BL) can be absorbed by the SBL1 tumour cells. We tested sera after four successive transplantations of these tumours and we failed to find any signs of agglutinin response.

E. Klein: Do you mean that you cannot get haemagglutinins by injection of the “original” tumour line? Or do you mean the “changed” line?

Feldman: Neither of them elicit haemagglutinins in C3H mice, no matter which procedure or which route of transplantation we use.

E. Klein: Do you see any difference in the quantity of absorption if you use both lines?

Feldman: We have published some data along these lines (Yaffe, D. and Feldman, M. [1959]. J. nat. Cancer Inst., 23, 132). The story is complicated because absorptions with the transformed line were done with tumours which were taken from the foreign host, whereas absorptions with the strain-specific lines were done with tumours which were grown in the strain of origin. Therefore the results are not clear-cut.

E. Klein: You might get around this problem if you injected both tumour lines in newborn animals of the same genotype.

Feldman: That could be done. However, what we have found is that the homotransplantable subline (SBLx), growing in hosts of foreign strains, does not absorb anti-C57BL isohaemagglutinins. Since these tumours were taken from genetically foreign hosts it might be thought that these tumours would be saturated with isohaemagglutinins. This seems very improbable, because for saturation you will need at least a degree of isohaemagglutinins which will be detected serologically.
E. Klein: You mean that the homotransplanted tumours have lost some antigen?

Feldman: Apparently, but this is not manifested in their susceptibility to transplantation immunity.

Lawrence: Could it be that with the hapten induction of the tumour you have covered the H-2 antigens, and in so doing have altered the "selfness" of that individual mouse by your hapten complex? This could cover the reactive groupings of what would be the H-2 agglutinin, and therefore interfere with induction of antibody formation, but not with the ability to absorb it. This has some particular bearing in view of Gell and Benacerraf’s (1961. J. exp. Med., 113, 571) recent findings (which had been postulated for homologous tissue antigens by Mitchison) that if simple chemicals are coupled to an individual’s own serum constituents this manoeuvre will alter the selfness of such materials. Gell and Benacerraf have shown that with simple chemical (picryl or p-chlorobenzoyl group) complexing of a guinea pig’s own or other’s serum albumin, for instance, it then functions as a foreign antigen for that host. I wonder how much of this sort of alteration may be contributing to the anomalous behaviour of your tumour homograft.

Feldman: Any of these possibilities might have happened. But since we do not know the antigenic structure of these antigens, it is hard to devise an experimental system to answer this question directly.

Loutit: When we injected sarcoma I, which is an A-strain tumour, into CBA/A radiation chimeras it produced (1) a little tumour, and (2) enormous enlargement of the lymph glands and spleen, a tremendous metastasis, so that superficially the animal appeared to have lymphomatosis. I understand that Dr. Feldman’s chimeras where foetal tissue has been given for restoration look the same. In our CBA mice given adult A bone marrow, the lymphoid tissue was completely replaced by hyaline fibrinoid material; the animals virtually lost their central mechanism of lymphoid tissue. If our CBA hosts are given foetal liver for restoration, we see a similar histological picture—complete absence of lymphoid tissue and a splenic pulp consisting of myeloid tissue. Dr. Feldman, you have discussed the afferent system and the efferent system. Have you any evidence of what is happening in the central system; is the lymphoid tissue intact?

Feldman: No, I haven’t. We did not really follow the morphological
appearance of these lymphoid tissues in relation to the tumours, but only tested for the existence of viable tumour cells by making cell suspensions of such organs and retransplanting.

Nakić: Dr. Loutit, are the animals suffering from the secondary disease immunologically incapacitated?

Loutit: Not entirely. But this is a very complex subject.

Nakić: We have had quite a different experience, although I am talking of a different experimental model—parabiosis between adult rats. We have found that animals tolerant of the cross-graft and dying of graft-versus-host reaction, with approximately the same histological picture you describe, can reject the graft from the third strain, although after some delay.

Woodruff: Dr. Feldman has shown that you can have tumour cells that have distributed themselves amongst lymph nodes and remain quiescent without developing metastases. We should remind ourselves that this is a fairly familiar phenomenon with spontaneous human tumours, and it is not confined to lymph nodes. This is illustrated by the following case. A patient who had had a melanoma removed three years previously from a leg, had a carcinoma of the breast treated in the orthodox way by a combination of surgery and radiotherapy, and within three weeks hundreds of thousands of melanomata appeared simultaneously in the field of irradiation. I think the only possible conclusion is that these cells were present for three years in the subcutaneous tissue but remained quiescent until the equilibrium between tumour and host was disturbed by the mechanical trauma of the operation, or the irradiation, or both.

Another point, following on what Dr. Loutit was saying, is that since irradiation (or administration of cytotoxic drugs) produces widespread damage to normal lymphoid tissue, it is desirable in treating patients with these agents to try to undo some of this incidental harm you have done, by taking out the patient’s spleen before starting treatment and putting it back intravenously in the form of a cell suspension afterwards. We have reported a number of cases in which we have done this (Woodruff, M. F. A. and Nolan, B. [1961]. Lancet, 2, 689).

Feldman: I fully agree with you. I think that the very fact that some type of resistance can be shown within the lymph nodes towards pre-existing tumour emboli tends to support your interpretation. The
point that we were trying to make is that such tumour emboli within the lymph nodes are much more susceptible to the host's response than the original tumour graft. This might apply also to metastasis of primary tumours.

*Amos:* I think it is very likely that transplanted tumours do carry antigens which are not particularly effective in eliciting immunity. When Gorer and I put E.L.4 into back-cross mice, the tumour killed rather more than half the back-cross, but if we gave them a little whole blood beforehand, most of the adults were able to reject the tumour. This corresponded to something like the presence of 5 antigens. So the tumour cells were in a condition in which they did not normally induce immunity but they were susceptible to it.

Secondly, we often found it quite difficult to show absorption of antibody with some of these non-specific tumours, especially the Ehrlich and the Ehrlich clones. Now it is often difficult to elicit isoantibody against these, but if one does, it can have quite a high titre. The tumour cells themselves are extremely inefficient at absorbing the antibody, and I have not seen the other sort of situation.

*Feldman:* As to the point about the capacity of such tumour cells to absorb isohaemagglutinins, one of the main differences between the examples which you have just quoted and these cases is that the Ehrlich tumour has long forgotten its genetic origin, whereas all these tumours with which we are dealing are recently established tumours which have been produced by benzpyrene and therefore would have much more of the original isoantigens than the type which you have described.

*Amos:* I would dispute this. Certainly the E-2 clone has remarkably remembered its origin: it does have a number of fairly characteristic antigens, which we can demonstrate are not derived from the strain in which it is being carried. The tumour has been passed through 20 transplant generations in DBA/2 and C3H hosts, and was still capable of giving virtually the same response in 129 mice.

*Feldman:* Even a partial loss of certain components among the various isoantigens will result in a lack of absorption, whereas complete absorption can take place only when all the components are there.

*Amos:* Another point is that some time ago Dr. Mirand and I did some experiments in which we transplanted DBA/2 lymphoma into the chorioallantoic membrane of chicks. After one chick embry
passage the tumours were very much less specific. We were not sure whether this was immunoselection or not. I am rather inclined to think it was not. The change was permanent.

Feldman: This alteration which you have obtained without selection is extremely interesting, particularly because we ourselves suspected in our cases even if there is any selective pressure it is not immunoselection.

Medawar: Could I make a suggestion arising out of McKhann’s work, and to some extent out of Lejeune’s? You might find that these changed tumours would sensitize if you trypsinized the cells. This seems to uncover antigenic potencies apparently not present before.

G. Klein: I have a comment which relates to the acquisition of homotransplantability after passage through newborn mice and perhaps also passage through chimeras. We have been working with some F₁ hybrid tumours, and both our group and Mitchison have found that if you have an F₁ hybrid tumour heterozygous for H-2, which can be symbolized, for the sake of simplicity by AS, then out of these tumours one can select variants compatible with A and variants compatible with S. If one takes one of these variants, for instance the one compatible with A, and tries to make it also compatible with S by passaging it through newborn hosts of the S type, sometimes it becomes homotransplantable and non-specific. But while the original variant formation and the development of the compatibility with the A parent involved the complete loss of the specific antigens of the S origin, to such an extent that they were no longer detectable by haemagglutination or by cytotoxic methods, the development of homotransplantability after passage through the newborn did not involve any further detectable loss of antigens and the H-2A system was still fully maintained. Therefore this second change is something entirely different from the first; it is not a further loss of antigenicity. I am wondering if there isn’t a phenomenon here that still eludes us, and that cannot be explained simply as loss of antigenicity.

Feldman: You are right.

G. Klein: You mentioned in your paper the effect of cortisone on the formation of metastases. I recently had occasion to review the literature on this point, and it appears that all authors who have shown that cortisone treatment has a promoting effect on metastasis formation
used either non-specific tumours, which means a genetically incompatible system, or else, if they used isologous systems they worked with long-transplanted tumours, like Baserga and his co-workers, for instance. In our laboratory, B. Törnberg carried out experiments of this type but with spontaneous tumours. He removed spontaneous mammary cancers operatively from C3H mice and divided the material into two groups. One group was treated with cortisone, while the other was left untreated. The frequency of lung metastases was compared in these two groups. Not only did cortisone not show any promoting effect upon metastasis in this system, but it had some slight inhibitory effect. In contrast, it had a promoting effect on a long-transplanted mammary cancer. I think it still remains to be shown whether the entire cortisone effect on metastasis is not due to an inhibition of homograft reaction. We do not perhaps yet have to postulate that the latent tumour cells that lie around in the lymph nodes and other tissues and seem to be promoted by cortisone treatment are necessarily latent because they are inhibited by an immune mechanism. The mechanism may be something other than an immunological one.

Feldman: It is certainly true that cortisone might do a number of things. For one thing, it might even activate the formation of metastases—not by means of suppressing any resistance—but due to the effect of cortisone on blood capillaries, which results in capillary arrest of tumour cells. This type of effect was recently reported by E. Zeidman (1961. Proc. Amer. Ass. Cancer Res., 3, 281). We have been studying tumours which apparently metastasize via the lymphatics. Whether the promotion of metastasis in chimeras, where the immune reactivity is decreased, is indeed due to suppression of some sort of resistance phenomenon, is still an open question.
THE FACTOR OF IMMUNIZATION: CLONAL SELECTION THEORY INVESTIGATED BY Spleen ASSAYS OF GRAFT-VERSUS-HOST REACTION

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Some two years ago, when the Ciba Foundation held its Symposium in Royaumont on Cellular Aspects of Immunity, a meeting generally influenced by the new theories on clonal selection, I closed my own contribution (Simonsen, 1960b) with the slightly nebulous prophecy that assays of graft-versus-host reactions (thinking particularly of spleen assays) would in the future help us to discriminate between the conflicting and highly speculative theories of antibody formation.

The attraction of graft-versus-host (GVH) reactions in this context is that some of them, at least, can be used as a means for quantitative estimation of immunologically competent cells. Hence they seem particularly fit for following the dynamics of cell populations of lymphoid organs during the course of immunization.

The past two years have in fact been witness of considerable interest in the use of GVH reaction for the study of clonal selection. Sir Macfarlane Burnet and his collaborators have made use of their chorioallantoic membrane (CAM) assay of GVH reaction. Their work, so far, has not provided unambiguous evidence in support of clonal selection. Indeed, one of their observations points rather strongly against it. This is the finding (Burnet and Burnet, 1960) that a grafted cell population, derived from a donor which
they have good reasons to believe was largely isogenic with the host, except for a single locus of a strong transplantation antigen, produced as many foci on the CAM as did similar cell suspensions from entirely unrelated donors. On their own hypothesis, the prediction would surely have been that the former donor, having only one clone of its immunologically competent cell population stimulated, would form fewer foci on the membrane than would the latter, which had many different clones being stimulated.

The present experiments, of which a preliminary account has been given by Simonsen (1962), were deliberately designed to challenge the hypothesis of clonal selection on a cellular basis.

The challenge consists in testing if specific immunization of the prospective donor for a subsequent GVH reaction does in fact lead to higher immunological competence per unit number of grafted spleen cells, as compared with normal donors. If this is actually found, it will only support the notion, which clonal selection shares with other theories of immunity, that antigenic stimulation leads to proliferation of antibody-forming cells, or their precursors. It would, however, have no bearing on the fundamental postulate in Burnet's theory, i.e. that the entire population of immunologically competent cells can be subdivided into clones with different predetermined affinities to different antigens.

If, on the other hand, it turns out that preimmunization of the grafted donor cell population makes it no more active than the non-preimmunized normal population, it would speak very strongly against the validity of clonal selection on a cellular basis. It would suggest that any immunologically competent member of the grafted normal cell population has in fact affinity to the antigen, provided it gets maximally stimulated, as it supposedly does by being grafted to a host containing the foreign antigen. Any proliferation which might have occurred during
preimmunization of the graft population would therefore lead only to a higher cell number, but not to a higher percentage of cells with a specific immunological reactivity.

The results indicate that some heightening of reactivity does occur in response to preimmunization with transplantation antigens of four other genotypes. But there are marked differences in the extent to which this occurs. Furthermore, there is an inverse relationship between the strength of the antigenic stimulus (as measured by running power) and the factor by which preimmunization increases the immunological reactivity of the immunized spleen. The results can formally be covered by a clonal selection hypothesis which differs radically from the one originally postulated by Burnet.

Material and methods

Mouse strains

The five strains employed in this investigation are, with their H-2 groups given in parentheses: C3H (k), DBA/2 (d), ST/A (b), A (a), and AKR (k).

General experimental design

C3H is used as the donor strain for the graft-versus-host reaction in infantile F1 hybrids between C3H and the four other strains. As the degree of spleen enlargement which results in this test system is, within a certain dose range, proportional to the logarithm of the number of grafted cells (Simonsen, 1962), it is possible to assay the relative potency of normal and preimmunized donors by injecting their spleen cells in graded dosages.

Technique of the spleen assay

Cell suspensions are prepared in Tyrode solution from the spleens of the two donors, one normal, and one preimmunized.
The two cell suspensions are adjusted, on the basis of preliminary experiments, so that their relative density can be expected roughly to compensate for their difference in potency. For example, if immune cells can be expected to be about 2–5 times as effective as normal cells, the latter suspension is always prepared three times as concentrated.

Most assays in the present investigation have been 6-point assays, i.e. the two suspensions have been tested in three dosages each. As the two suspensions are naturally best compared in one and the same litter, it means that only litters of seven or more members are suitable test objects, number 7 (and possibly also 8) serving as controls injected with isogenic spleen. A litter size over 8 is always reduced to that number, as bigger litters often tend to be malnourished. Obvious congenital runts are discarded before injection.

The doses of each suspension are always spaced logarithmically to facilitate later computation (cf. Table I).

If, as in Table I, the highest dose injected into any litter member is 9 million cells, all other doses are made up to 9 million by the addition of the required number of inert cells; for which purpose spleen cells from an adult F₁ hybrid, isogenic with the test litter, are used. The reason for doing this is that (unpublished) experiments, together with Dr. D. Michie, have indicated that a mechanical saturation effect may be operating if the titration is not done in a constant total cell number. It is easy to visualize that an infantile mouse spleen will accommodate a decreasing fraction of the injected cells as the dose is increased. By observing the condition of a constant total cell number it is supposed that the same fraction of the inoculum settles in the spleen in all animals, so that the ratio between dose levels as prepared in vitro will actually be maintained in vivo.

Test litters are killed 8–10 days after the injection, and spleen indices determined as described earlier (Simonsen 1962, and previous publications referred to there).
Table I

The factor of immunization determined in C3H→C3H × ST/A. Full data from an experiment involving 7 pairs of immune and normal donors tested in 12 infantile hybrid litters.

<table>
<thead>
<tr>
<th>Test litter</th>
<th>Immune donor</th>
<th>Total spleen cell count</th>
<th>Spleen indices (cell dose in million)</th>
<th>Factor of immunization (F.I.)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Age in days</td>
<td>Days after no. of injections</td>
<td>Ratio I/N*</td>
<td>I₁ (1/3)</td>
</tr>
<tr>
<td>1277</td>
<td>8</td>
<td>7 after 1</td>
<td>1.36</td>
<td>1.17</td>
</tr>
<tr>
<td>1265</td>
<td>1</td>
<td>12</td>
<td>1.38</td>
<td>1.14</td>
</tr>
<tr>
<td>1252</td>
<td>2</td>
<td>16</td>
<td>1.11</td>
<td>1.13</td>
</tr>
<tr>
<td>1253</td>
<td>4</td>
<td>same donor</td>
<td>1.85</td>
<td>2.76</td>
</tr>
<tr>
<td>1250</td>
<td>5</td>
<td>16</td>
<td>1.30</td>
<td>1.00</td>
</tr>
<tr>
<td>1251</td>
<td>2</td>
<td>same donor</td>
<td>1.25</td>
<td>1.58</td>
</tr>
<tr>
<td>1239</td>
<td>3</td>
<td>9</td>
<td>1.26</td>
<td>0.97</td>
</tr>
<tr>
<td>1240</td>
<td>2</td>
<td>same donor</td>
<td>1.14</td>
<td>1.82</td>
</tr>
<tr>
<td>1182</td>
<td>4</td>
<td>12</td>
<td>1.21</td>
<td>1.13</td>
</tr>
<tr>
<td>1183</td>
<td>4</td>
<td>same donor</td>
<td>1.11</td>
<td>1.61</td>
</tr>
<tr>
<td>1217</td>
<td>3</td>
<td>8</td>
<td>1.20</td>
<td>1.31</td>
</tr>
<tr>
<td>1219</td>
<td>1</td>
<td>same donor</td>
<td>2.01</td>
<td>2.40</td>
</tr>
</tbody>
</table>

N = 12

Sum (S)  8.82
Mean (M) 1.26
Standard error of mean (s.e.m.) 0.03

* Calculated according to the formula log F.I. = \( \bar{x}N - \bar{y}I - \frac{\bar{y}N - \bar{y}I}{b} \)

where \( \bar{x} \) and \( \bar{y} \) represent mean log dose and mean spleen index of animals treated with normal (N) or immune (I) cells.
Preimmunization of donors

The immune C3H donors have all been injected intraperitoneally with spleen cells from F1 hybrids which were isogenic with the prospective test litter.

There are two reasons for not using foreign pure-strain cells as antigen source: (1) they might possibly survive in the prospective C3H donor's spleen long enough to give a graft-versus-host reaction of their own, after injection into the infantile F1 hybrid; and (2) they might also produce a graft-versus-host reaction in the C3H mouse, which fact, in consequence, might interfere with the process of immunization, especially in hyperimmunized mice (cf. Brent and Medawar, 1962). The doses used for immunization have been \( \frac{1}{2}-1 \) hybrid spleen per injection. Repeated injections have always been spaced at least 1 week apart; usually 3 weeks have elapsed between the first and the second.

Results

Factor of immunization in response to ST/A antigen

This will be mentioned first because a full account of the data (Table I) together with the graphical distribution of the mean values of the spleen indices (Fig. 1) serve to illustrate the meaning of the term, Factor of Immunization (F.I.).

The bottom of Table I gives the sums and means of the spleen indices for the 6 points of titration, 3 points representing normal (N), and 3 points immune (I) donors.

The means are plotted in Fig. 1 against the log doses employed, and 2 straight lines have been drawn in parallel to fit the data. The slope and position of these lines have been determined as follows.

The common slope is based on the assumption that all differences in observed slopes from one test litter to another as well as between normal and immune curves within the same litter are all due to sampling error. Hence, they are all more or less imperfect terrestrial copies of the true platonic slope for this
strain combination. The best approximation we can get is, therefore, the "semi-platonic" slope which is determined by pooling all top dose indices from the 2 sets of data and subtracting the pool of bottom dose indices, and dividing the result by 2 times the number of test litters multiplied by the dose range (which is log 9 in both curves). Therefore the formula for the slope is:

\[ b = \frac{SI_3 + SN_3 - (SI_1 + SN_1)}{2 \times 12 \times \log 9}; \]

\( b \) equals in this experiment 1.32.
Having calculated the slope, the position of the 2 parallel lines is determined by the mean dose and mean response for the normal curve, and similarly for the immune one.

Readers interested in the statistical theory of parallel-line assays may refer to the textbook by Finney (1952).

We now come to the question of calculating the Factor of Immunization, i.e. the potency ratio between immune and normal cells. This factor is the horizontal distance between the 2 parallel lines. No matter where the horizontal line is drawn, this distance is obviously the same and represents, for a given response,

$$\log \text{dose } N - \log \text{dose } I = \log \frac{\text{dose } N}{\text{dose } I},$$

The antilogarithm to the distance gives therefore (dose $N$/dose $I$), the Factor of Immunization, indicating how many more normal cells than immune cells are needed to give the same degree of spleen enlargement.

As seen from Fig. 1, where the horizontal distance has been drawn through the point of mean response on the immune curve, the distance is easy to calculate as the sum of $AD$ and $DB$. $AD$ is simply the difference between the mean doses employed (measured on the logarithmic scale); that is, in this experiment, $\log 3 - \log 1 = \log 3$. $DB$ is a little more laborious to calculate, but it only amounts to dividing $DC$ (the difference between mean responses) with the earlier calculated slope $b$. This is easily seen from the triangle BCD, where $(DC/DB) = \tan \nu$ (= the angle of the parallel lines with the $x$-axis); the tangent to $\nu$ being the definition of the slope.

Calculation of the Factor of Immunization can be made on a purely arithmetical basis, according to the general formula

$$\log \text{F.I.} = \bar{x}N - \bar{x}I - \frac{\bar{y}N - \bar{y}I}{b},$$

where $\bar{x}$ represents the mean log dose and $\bar{y}$ the mean response (Finney, 1952).
If we apply this formula to the mean values of Table I (or Fig. 1) we will get

$$\log \text{F.I.} = \log 3 - \frac{(1.27 + 1.74 + 2.52) - (1.27 + 1.97 + 2.54)}{3 \times 1.32}$$

$$= 0.477 + \frac{0.25}{3.96} = 0.540, \text{ or F.I.} = 3.5.$$ 

This figure, as seen from Table I, is an estimate based on pooling the results from experiments in which the history of the immune donor varies from having been used at 7 days after 1 injection to 8 days after 5 injections. While this procedure is fully legitimate for calculation of the common slope, $b$, it is, nevertheless, wasteful of information.

We also want to know if there is any variation in the F.I. depending on the schedule of immunization. Tentatively, the F.I. has been calculated for each test litter (inserting the corresponding mean responses in the formula above, and using the "semi-platonic" slope throughout). These figures are entered in the last column of Table I. Apart from a single test litter (1217) there is no more than a rather feeble suggestion that the F.I. is dependent on the number of immunizing injections. The high factor given by litter no. 1217 is most likely to be due to an exceptionally great sampling error, all the more so since parallel titration of the same suspension in litter no. 1219 gave a F.I. within the normal range.

The procedure of determining the F.I. for each individual litter is not, however, the best way to treat the data—not even if they were obtained from assays with fully comparable conditions. The sampling error is just too big, as illustrated above by the test litters no. 1217 and 1219.

It seems more reasonable to subdivide the titrations of Table I into two groups according to whether the immunized donor has received a single or repeated injections, and thereafter treat each group as a whole. The group representing single injections
MORTEN SIMONSEN

consists of 4 immune donors. Two of the donors have been tested in 2 litters, but in these cases the mean values of the 2 litters are used, so that each immunized donor (and its normal control donor) is represented by one set of data only.

This leaves us with a block of spleen indices analogous to Table I except that it consists of only 4 horizontal columns. These columns are now summed vertically, as was done with all the data of Table I. The F.I. is then calculated on the basis of the mean values, whereby sampling errors of individual test litters largely cancel each other out. The group of repeatedly injected donors is treated in the same manner. The results are entered in Table II together with similar results obtained from the 3 other strain combinations which have been studied.

The C3H×AKR F1 hybrids have presented special difficulties which stem from the fact that normal C3H cells, even in very high doses, only produce a small degree of spleen enlargement (almost invariably an index below 1.50). The actual procedure adopted for this combination (in contrast to the three others which followed the pattern described for the combination with ST/A) was to make a 3-point titration with preimmunized C3H cells (doses: 0.5, 1.5, and 4.5 million), and a 1-point determination of the response to normal C3H cells (20 million). The immune cell doses were of course supplemented to a total of 20 million by the addition of inert cells. The F.I. was eventually determined as the horizontal distance between the immune line and a line drawn parallel to it, through the mean response to 20 million normal cells. The assumption of parallelism in this combination rests therefore entirely on analogy with the 3 other combinations, where the difference in slopes between normal and immune curves did not deviate significantly from zero.

**Comparison of F.I. in 4 strain combinations**

Table II, column 5, shows clearly that the F.I. differs in C3H mice in response to the 4 foreign strains which have been tested.
## Table II

**Factor of immunization found after single and repeated injections of C3H mice with cells of four different strains**

<table>
<thead>
<tr>
<th>Foreign antigen</th>
<th>History of immune donors</th>
<th>Number of donor pairs</th>
<th>Test litters</th>
<th>Total spleen cell count</th>
<th>Factor of immunization</th>
<th>No. of C3H spleen cells to give spleen index = 2.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>1–2 wk. after 1 injection</td>
<td>3</td>
<td>11</td>
<td>1.29 ± 0.08</td>
<td>1.7</td>
<td>b</td>
</tr>
<tr>
<td>DBA/2</td>
<td>1–2 wk. after 2–4 injections</td>
<td>5</td>
<td>6</td>
<td>1.14 ± 0.15</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>ST/A</td>
<td>1–2 wk. after 1 injection</td>
<td>4</td>
<td>6</td>
<td>1.29 ± 0.03</td>
<td>2.5</td>
<td>0.8</td>
</tr>
<tr>
<td>ST/A</td>
<td>1–2 wk. after 3–5 injections</td>
<td>3</td>
<td>6</td>
<td>1.11 ± 0.10</td>
<td>5.0</td>
<td>3.7</td>
</tr>
<tr>
<td>A</td>
<td>1–5 wk. after 1 injection</td>
<td>2</td>
<td>3</td>
<td>no counts</td>
<td>7.3</td>
<td>0.7</td>
</tr>
<tr>
<td>A</td>
<td>2 wk. after 2–4 injections</td>
<td>3</td>
<td>4</td>
<td>1.31 ± 0.10</td>
<td>18.0</td>
<td>45.0</td>
</tr>
<tr>
<td>AKR</td>
<td>1–2 wk. after 1 injections</td>
<td>2</td>
<td>3</td>
<td>1.07 ± 0.07</td>
<td>8.3</td>
<td>168.0</td>
</tr>
<tr>
<td>AKR</td>
<td>1–2 wk. after 5 injections</td>
<td>2</td>
<td>4</td>
<td>2.07 ± 0.22</td>
<td>66.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* These figures include a few counts from preliminary experiments, not otherwise included in this paper.
The four strains can be ranked in order of DBA/2, ST/A, A and AKR, beginning with the lowest values.

The differences are particularly marked when hyperimmunized mice are compared. Regarding the F.I. after a single immunizing injection, there is still a significant difference between DBA/2 and ST/A on the one hand, and A and AKR on the other hand.

Column 6 of Table II indicates the numbers of normal C₃H spleen cells which, on the average, are necessary to produce a spleen index of 2·00. These figures have been estimated by interpolation from the straight lines determined by the respective “semi-platonic” slopes and the points of mean dose-response for all titrations of normal cells (the N-curve of Fig. 1 for the injection of C₃H cells into C₃H x ST/A F₁ hybrids).

By dividing the figures of column 6 by their corresponding F.I. as determined in hyperimmunized donors (column 5), the figures of column 7 are obtained, indicating how many hyperimmune cells are, on the average, needed to produce the same spleen enlargement. It is quite apparent that the great bulk of variation in the F.I., if not the whole, resides in the difference with which normal C₃H cells react upon different antigens. It is remarkable how similar the figures are, in column 7, for DBA/2 and ST/A, and likewise for A and AKR. Exactly the same statement applies to column 8 which gives the “semi-platonic” slopes.

**Antigenic strength in terms of runting power**

It is well established that splenomegaly is a characteristic sign in the early phase of runt disease. It follows, therefore, that column 6 of Table II, which gives the estimated numbers of normal C₃H spleen cells necessary to produce a doubling of the spleen weight in the 4 different F₁ hybrids with C₃H, gives an indication of the relative strength which the 4 different strain antigens represent vis à vis C₃H. It appears that DBA/2 represents the strongest antigen, followed by ST/A, A and AKR, in this order.

It was, nevertheless, thought desirable to investigate whether
the cardinal signs of overt runt disease, weight retardation and death, would establish the same ranking of antigenic strength.

For this purpose, members of 0–1-day-old F₁ hybrid litters were each injected i.p. with 25 million adult C₃H spleen cells. One, or usually, two litter-mates were kept as non-injected controls. The period of observation was 30 days, as death would have usually occurred by then if at all. Mice which died before day 5 after injection were discarded as having probably died from reasons other than runting. All litters were inspected daily for recording of deaths, and all survivors at day 21 were weighed on that day.

The dose of 25 million C₃H cells was chosen because earlier experiments (with a different purpose) had already suggested that this dose produced a higher mortality in C₃H hybrids with DBA/₂ than in similar hybrids with ST/A.

It has been a great weakness in the utilization which has been made so far of data for death and weight retardation in runt disease, that no attempt has been made to combine the two kinds of information in a single formula.

When a less than 100 per cent lethal dose is injected, the characteristic outcome will necessarily be that some litter-mates show more or less weight retardation, while others die at different times. How is the combined result from such a litter to be expressed numerically?

Table III represents a crude attempt at solving this problem, and gives, at the same time, the data for that litter in the series with the severest runting compatible with a life-span corresponding to the period of observation. This animal had, at day 21, a body weight which was 40 per cent of its normal control, or a ratio of 0·40.

We shall now assume that it was a matter of sheer luck that this mouse was still alive at day 30. If we equilibrate its degree of runting to that of a hypothetical litter-mate which would have died at day 30, both mice would be given an index of runting of 0·40.
Table III

Index of runting. Specimen litter demonstrating the calculation of the index. The litter is newborn (C3H×DBA/2) F1 injected with 25 million adult C3H spleen cells

<table>
<thead>
<tr>
<th>Litter member (Treatment)</th>
<th>Day of death</th>
<th>Body weight at day 21 (g.)</th>
<th>Index of runting*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (25×10⁶ spleen cells)</td>
<td>14</td>
<td>9/25×0.40 = 0.36</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>16/25×0.40 = 0.64</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>16/25×0.40 = 0.64</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>23/25×0.40 = 0.92</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>&gt;30</td>
<td>Ratio = 4.8/12.0 = 0.40</td>
<td></td>
</tr>
<tr>
<td>6 (non-injected)</td>
<td>&gt;30</td>
<td>Mean = 0.29</td>
<td></td>
</tr>
</tbody>
</table>

* For animals dying at time t between days 5 and 30, this index is expressed as (t−5)/(25)×0.40. If the test animals survive the period of observation, the index is determined as the body weight ratio between test animals and litter-mate controls at day 21.

We next want to construct a single scale of indices which extends from 0.40 to 1.00 for milder degrees of runting, and from 0.40 to 0 for more severe runting. The extension upwards is easy. It comprises survivors only, and these are given the index determined by the ratio of their body weight, at day 21, relative to normal litter-mates. There are no such animals in Table III.

The extension downwards requires that an animal which dies at day 29 gets a lower index than 0.40, and that the lowest possible index is given by a mouse which dies at day 5. We may define the index of runting in these cases by the equation: index of runting = (t−5)/(25)×0.40. This gives an index of 0 for a mouse dying at the 5th day, and an index of (25)/(25)×0.40 = 0.40 for a mouse dying at day 30.

It is hardly necessary to emphasize that this is purely an *ad hoc* procedure for the purpose of the present experiments. No attempt has yet been made to determine if this manner of expressing severity of runting would withstand the requirements of a dose-response assay.

The data of Table IV show the indices of runting, as determined
Table IV

Severity of runt disease caused by i.p. injection of 25 million adult C\textsubscript{3}H spleen cells into newborn F\textsubscript{1} hybrids of C\textsubscript{3}H with four other strains (DBA/2, ST/A, A and AKR)

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>DBA/2</th>
<th>ST/A</th>
<th>A</th>
<th>AKR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(age of recipient at injection)</td>
<td>Death rate*</td>
<td>Index of running†</td>
<td>Death rate</td>
<td>Index of running</td>
</tr>
<tr>
<td>I (24–48 hr.)</td>
<td>5/5</td>
<td>0.18</td>
<td>4/7</td>
<td>0.41</td>
</tr>
<tr>
<td>II (0–24 hr.)</td>
<td>9/9</td>
<td>0.19</td>
<td>11/11</td>
<td>0.20</td>
</tr>
<tr>
<td>III (0–24 hr.)</td>
<td>5/5</td>
<td>0.15</td>
<td>0/10</td>
<td>0.91</td>
</tr>
<tr>
<td>IV (24–48 hr.)</td>
<td>2/2</td>
<td>0.19</td>
<td>17/17</td>
<td>0.19</td>
</tr>
<tr>
<td>V (24–48 hr.)</td>
<td>16/17</td>
<td>0.21</td>
<td>3/11</td>
<td>0.54</td>
</tr>
<tr>
<td>Total:</td>
<td>32/33</td>
<td></td>
<td>40/51</td>
<td></td>
</tr>
<tr>
<td>Mean:</td>
<td>0.19</td>
<td></td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

* Number of animals dead within 5–30 days after injection divided by number of injected animals.
† Index of running calculated as described in the text and in Table III.
by this method, in the 4 strain combinations with C3H. It is
evident that they indicate a relative antigenic strength which
conforms with the estimates on spleen enlargement (Table II,
column 6).

Discussion

The present experiments compared with earlier findings

Other workers have provided evidence that preimmunized
donors provoke a stronger graft-versus-host reaction than normal
donors (e.g. Billingham and Brent, 1959; Gorer and Boyse, 1959;
Howard, 1961). However, their assays do not allow conclusions
to be drawn about the relative potency of the two kinds of cells.
Nor do they suggest any difference between weaker and stronger
antigens in this respect.

The latter fact, however, is strongly suggested by the studies
of Berrian and McKhann (1960) who performed 1st- and 2nd-set
skin grafts in co-isogenic strains of mice differing either in respect
to the H-2 or the H-3 locus. When the antigen was a strong one
(H-2), the 2nd-set grafts survived only 3 days less than the 1st-set
(6 days instead of 9). Whereas, when the antigen was weak (H-3),
2nd-set grafts lasted 8 days as contrasted to 20 or 34 days in the
two strain combinations tested.

The skin grafting technique has very severe limitations for this
kind of study. The short survival of 1st-set grafts in a strong
combination sets a very narrow limit to the degree of immunity
which can be detected here. Even if the gain in “rejecting power”
was equal to, or greater than in a weak system, there would be
no way of observing it. With these reservations in mind, there is
clearly agreement in qualitative terms between the findings of
Berrian and McKhann and the present findings.

The present experiments in relation to clonal selection

There is much in the discussion in my recent review on graft-
versus-host reactions (Simonsen 1962, chapter X) that would be
relevant here. Repetition, however, cannot be entirely avoided.
In every version of clonal selection theory of immunity, it is assumed, (1), that the capacity for immunological reactivity (for convenience often referred to as antibody formation) is genetically predetermined in the cell, and (2) that it is by virtue of this pre-existing, though low-grade reactivity, that the antigen is recognizable to the immunologically competent cell. In Burnet’s views on clonal selection, the whole population of immunologically competent cells can be subdivided into clones, each of which has a limited number of potentialities for reaction to foreign antigens. As the number of different antigens which any organism can be confronted with is enormous, each clone is consequently supposed to form an initially small fraction of the entire population. It is only by the introduction of an antigen that the corresponding clone will be selectively stimulated to proliferate and come to form a substantial part of the entire population.

In the words of Burnet and Burnet (1960): “antibody production is presumed to occur in two phases corresponding (1) to the inductive phase and (2) to the production phase of orthodox immunological theories. In the induction phase two processes occur; there is proliferation of the clone to produce many more members, and the newly produced cells have an increased reactivity with the corresponding antigenic determinant. In the second phase, renewed contact with the antigenic determinant results in proliferation, conversion to the plasma cell form and production of antibody”.

What constitutes the exact mechanism of the proliferative stimulus seems irrelevant in this context. The main points are that proliferation is compelling if antibody production of the clone is to be increased from its usually sub-detectable level to one which is easily measurable (whether in terms of cellular or humoral immunity), and that this proliferation brings the clone to occupy a much higher proportion of the total population of immunologically competent cells.

Surely, in the CAM assay system, Burnet and co-workers
should expect a specifically preimmunized donor to produce a higher number of foci on the membrane than would a non-preimmunized donor (an experiment, which, to my knowledge, has not yet been carried out). Likewise, in the spleen assay system, fewer immune cells than normal cells would be expected to produce the same degree of spleen enlargement (i.e. a high Factor of Immunization).

As the results have clearly shown, this expectation is fulfilled very well for immunization of C3H with AKR antigen, less well with A antigen, rather poorly with ST/A antigen, and hardly at all with DBA/2 antigen. It is of course the data of Table II for hyperimmununized donors which are most relevant here, as these are the ones where the stimulated clones have had the best opportunity for expansion.

If we assume, as Burnet and Burnet also did in the above quotation, that active immunization also leads to a higher reactivity per cell, there seems virtually no space left for clonal selection in the combination with DBA/2.

It seems to me, however, that the results can still be very well accommodated into a clonal selection frame of thought if the hypothesis is formulated as follows.

(a) All immunologically competent cells in the same individual are originally endowed with low-grade reactivity (affinity) to all antigens (essentially Lederberg’s idea of clonal selection on an intracellular basis, 1959).

(b) In the process of ontogeny, affinities to self-components are eliminated or suppressed through a process of overloading with antigen (Burnet, 1959).

(c) Throughout the life of the individual, mutational losses occur on the various genetic loci which are determining the inborn immunological affinities of the cells. This leads to differentiation in cellular clones, some of which have lost some affinities, whilst others have lost other affinities.

(d) The rate at which loss-mutations occur is very different for
different loci, which fact might reflect that immunological reactivity is more essential for the organism in respect to some antigens than to others. Some loci would never lose their active genes. If so, the antigens against which the activity of these genes is directed would be obligatory in the sense that all immunologically competent cells would react against them, given optimal conditions for stimulation by the antigen. Other antigens would be more or less facultative in the sense that the genes which determine reactivity against them have been lost in a smaller or greater part of the entire cell population.

Facultative antigens, upon their injection, would therefore lead to clonal selection in the sense which Burnet originally visualized, while obligatory antigens would not. Fig. 2 is intended to represent a crude illustration of this hypothesis as applied to the four antigens of the present investigation. The cells I-IV symbolize

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**Fig. 2.** Clonal selection on basis of loss mutations.
members of different clones. Clone I is supposed to possess all genes necessary for production of antibodies to all 4 antigens. Clone II has lost genes necessary for reaction to AKR antigen, while clones III and IV represent further losses. Reactivity to DBA/2 antigen, however, remains intact even in clone IV.

If the C3H mouse (being the owner of all these clones) is injected with DBA/2 antigen, there is no reason why one clone would be stimulated to proliferate more than the others. The percentage of cells with affinity to DBA/2 would of course remain at 100 as it was before stimulation, and the small increase in the F.I. (to about 1.8) could be ascribable to a higher reactivity per cell.

The extreme alternative situation is the one in which AKR antigen is injected (as supposed in Fig. 2). In this case only clone I could be stimulated. The proliferation which this would lead to, would raise the percentage of cells with affinity to AKR (presumably even more so if hyperimmunization was continued). As the result of proliferation of clone I, it would also be expected that reactivity to facultative antigens other than AKR would be increased at the same time if the genes which determined reactivity towards them happened to be present in the same clone. This would in fact be a possible mechanism for "non-specific" stimulation of immunological defence reactions, as opposed to that which results when there is a sharing of common antigens.

The main purpose of Fig. 2 is, however, merely to illustrate the notion that different rates of mutational losses in different loci of immunologically competent cells will lead to different proportions between clones when they are stimulated by their corresponding antigens.

It may be of significance in this context that the total number of spleen cells recovered from the hyperimmunized C3H spleens, as compared to normal C3H spleens (Table II, column 4), was, on the average, increased much more after injection with AKR antigen than after any of the other antigens. The donors which
had received a single immunizing injection showed a slightly increased total cell count but, if anything, rather less after immunization with AKR than after the two strongest antigens.

The present experiments in relation to acquired tolerance

Although these experiments seem to deal exclusively with immunity, there is nevertheless one finding which I have been unable to explain except in terms of tolerance. This is the fact (Table II, column 8) that the "semi-platonic" slopes in the 4 strain combinations apparently fall in two categories, one slope for the 2 stronger antigens, and another lower one for the 2 weaker antigens.

This difference seems to be more than a sampling error. An analysis of variance performed on the 4 slope populations has shown that the 2 steeper ones are insignificantly different, and so are the 2 smaller ones, while any of each group is significantly different from any in the other group.

I would not maintain that this fact is an obvious sign of tolerance. But, before trying to evolve how it might be suggestive, I will stress the view which I think all workers in transplantation biology share, that tolerance is easier to induce with weak than with strong antigens. This fact is well illustrated in parabiosis experiments with inbred mouse strains where a condition similar to tolerance (at least superficially) can be achieved (see Martinez, Shapiro and Good, 1960, 1961).

Returning to the slope differences, it could be argued that if tolerance is induced in a substantial part of the grafted C3H cells before they have settled in the spleen, it will lead to reduction of their spleen enlarging capacity; furthermore, that this reduction might be percentually higher for a large dose than for a small dose, in which case it would lead to reduction of the slope, in addition to parallel displacement of the curve.

Simonsen (1962) has previously presented data to suggest that C3H cells injected i.p. into (C3H × ST/A) F1, which forms one of
the “strong” test systems employed in the present work, may be rendered highly tolerant within 24 hours after grafting. If this is so, tolerance is probably also induced in the weaker test systems, and presumably even faster.

The possibility can therefore not be dismissed that part of the grafted cell population is rendered tolerant before it has succeeded in producing the damage necessary for production of splenomegaly. If, however, the assumption is correct that tolerance in grafted cell populations is induced faster in response to weak than to strong antigens, this fact might furnish an alternative explanation of the different Factors of Immunization.

It could now be postulated that:

(a) There need not be a clonal selection on a cellular basis; all cells may maintain potential reactivity to all antigens provided they are adequately stimulated, as they are presumed to be in a graft-versus-host reaction.

(b) Strong antigens do not allow production of tolerance against them in the time required by the grafted cells to be stimulated and hence to initiate a graft-versus-host reaction. Virtually nothing is gained, therefore, by preimmunizing the donor; hence a low F.I.

(c) Weak antigens, on the contrary, induce a considerable degree of tolerance in the graft. A larger dose of normal cells has to be injected in order to obtain a similar degree of spleen enlargement (cf. Table II, column 6).

(d) Preimmunization of the donor renders the cells relatively refractory to the induction of tolerance (of which I have some independent, but incomplete evidence). While this is immaterial in a strong antigenic combination, it allows the graft in a weak combination to preserve its reactivity until the spleen has been damaged; hence a high Factor of Immunization.

With either explanation, it is assumed that (1) the graft-versus-host reaction which results in splenomegaly takes place in a relatively short time (probably mostly within the first day, cf. Simonsen, 1962), and (2) that the cells which cause splenic
enlargement by their proliferation are mainly cells of the host, as Davies and Doak (1960) have shown to be true in the mouse.

The choice between hypotheses

I have offered both explanations of the observed differences in my study of the Factor of Immunization, as I am unable for the moment to decide which, if either, is more likely to be correct.

The "tolerance" explanation has the unattractive side to it that tolerance itself is left entirely unexplained.

The hypothesis of clonal selection on the basis of loss mutations has, in contrast, the virtue that it at least helps to explain how tolerance can be achieved more easily with weak than with strong antigenic combinations. If an antigen is weak because cells with affinity for it are numerically small to begin with, it is obviously easier to overload them with antigen to such an extent that their reactivity becomes abolished or suppressed.

In strong combinations, on the other hand, the antigenic load will have to be higher and probably of longer duration. The chances of achieving tolerance in this situation may depend on the possibility that some clones may exist, or arise, which do not have affinity to an otherwise obligatory antigen. When the antigenic load is of sufficiently long duration (as in parabiosis of parental strain with $F_1$ hybrid), these exceptional clones will be favoured by a selection pressure, provided exhaustive sensitization (Simonsen, 1960a) ultimately inactivates the reacting clones. Admittedly, exhaustive sensitization could equally well be regarded as favouring the growth of tolerant cells arising from stem cells with no genetically predetermined affinity. I believe, however, that I have recently provided some rather suggestive evidence against the stem cell hypothesis (Simonsen, 1962), and would, therefore, prefer to regard the development of tolerance as having some basis in the heterogeneity of the population of immunologically competent cells rather than in postulated differences between stem cells and differentiated cells.
It should be noted that the two hypotheses I have offered as explanations of my present findings are not mutually exclusive. The "loss-mutation" hypothesis provides, at the same time, a partial explanation of the "tolerance" hypothesis, in so far as it accounts for the greater ease with which tolerance is induced in the various strain combinations. Consequently, it also accounts for the differences in Factors of Immunization. Alternatively, if the uneven inducibility of tolerance has a different and, to my knowledge, quite unsubstantiated mechanism, there may be no need to assume a "loss-mutation" hypothesis in order to explain the different Factors of Immunization.

Whatever may represent "the truth" in the realm of immunological theory, it seems very clear that "antigenic strength" in transplantation biology is primarily dependent on the intensity of the initial reactivity to the antigen. Given sufficient time to provoke the immune response, antigens are not very different in strength.

**Summary**

The term Factor of Immunization (F.I.), is introduced to signify the potency ratio between 2 suspensions of immunologically competent cells, one derived from a normal animal, and the other from a preimmunized animal.

The reactivity of the two cell suspensions is measured by the spleen assay of graft-versus-host reaction.

The F.I. has been measured in four strain combinations with C3H as the donor strain. The four kinds of recipients used in the spleen assays were F₁ hybrids between C3H and DBA/2 (H-2^d), ST/A (H-2^b), A (H-2^a) and AKR (H-2^k).

The antigenic strength which these foreign strains represent vis à vis C3H has been measured in two ways: by the number of normal C3H cells needed to give a doubling of the spleen weight in the corresponding F₁ hybrids, and by the degree of runting which results from injection of 25 million normal C3H cells.
The results indicate that the four strains can be ranked in the order DBA/2, ST/A, A and AKR in respect to increasing F.I., whereas the order is reversed in respect to antigenic strength.

The findings are discussed in relation to clonal selection theories of immunity and tolerance. A new version of the clonal selection hypothesis is proposed as the most satisfactory explanation for the findings.

Irrespective of the theoretical interpretation, the main conclusion is that “antigenic strength” in transplantation biology is primarily dependent on the initial reactivity to the antigen. Given sufficient time to provoke the immune response, antigens are not very different in strength.

REFERENCES

DISCUSSION

Medawar: Could I propose what seems, superficially perhaps, to be an alternative interpretation of the difference between the strong and the weak antigen?—that the relatively weak antigen is an antigen in respect of which the ability of a host cell to respond matures relatively late in life? This is making the distinction between strong and weak antigen turn upon chronological rather than purely numerical matters. This interpretation would explain the empirical fact that the weaker an antigen, in the transplantation system, the later in life is it possible to induce tolerance. You needn’t assume that the population of responding cells is heterogeneous; all you have to assume is that competence to respond to different antigens matures at different times.

Simonsen: If you take the induction of tolerance in adults by the parabiosis technique in which you parabiose with F₁ hybrid, I suppose on your assumption it should have had time enough to develop reactivity towards even the weakest antigens, since these are fully adult mice. But the difference between the strong and the weak antigens still remains; it is much easier to induce tolerance towards the weak antigens than to the strong.

Medawar: That is what I would expect in the adult. According to McKhann’s recent results on co-isogenic strains differing at H-3—whether you get immunity or tolerance varies with the dosage of spleen cells: a low dose of cells will excite immunity but a higher dose very easily excites what one could well describe as tolerance, though I don’t think the analysis has gone far enough to show whether it is tolerance or not. One ought not to forget that there is a special sense in which antibody-forming cells might form a heterogeneous population: they may be heterogeneous in the sense that different immunological faculties mature in those cells at different times. Is this idea eliminated by anything we know?

Michie: Are you suggesting that in the adult mouse there is complete failure of maturation towards some weak antigens—even when the mouse is “middle-aged”?

Medawar: No. I was wondering whether it was possible to think that in an adult mouse only a relatively small proportion of the cell popul-
 aroun had matured to a degree at which they could respond to H-3 antigens.

Michie: How does it measure up to this point, that the difference between the immunizing and tolerance-inducing dose doesn’t seem to change markedly with age? The difference between the tolerance-inducing dose for a strong (H-2) antigen and a weak one is just about the same in adults as it is in the newborn, that is of the order of 10 or 100 times. And if you compare the Y antigen with an H-2 difference, I think you get a factor of the order of at least 100 for equivalent tolerance-inducing dose. But that factor is the same in newborns as it is in adults.

Medawar: Yes, I see the point.

Mitchison: Dr. Simonsen, in the second part of your exposition I understood you to interpret the Factor of Immunization as being a measure of the extent of the multiplication of the population of cells. Might it not equally be that this represented a heightened reactivity on the part of cells that did not multiply—that each individual cell becomes relatively more potent as a consequence of immunization? And so discussions of clonal selection in that context might be irrelevant.

You mentioned quantitative immunochemistry and I am trying to translate the data which you have given here into the form in which they would appear if one were doing a classical immunization experiment. If I have understood you correctly one could translate them into three propositions, which I think are very well established already, namely: (i) that over at least a proportion of the dose-response range the amount of antibody made is proportional to the amount of antigen put in; (2) that if you compare different antigens, they elicit different quantities of antibody during the primary response, and that those antigens which give a powerful initial stimulus tend to cause a less marked secondary response (contrast the bacterial flagellar proteins with heterologous plasma proteins: the former are more powerful initial immunogens and give less of a secondary response than the soluble proteins); (3) that rabbits tend to make antibodies up to some sort of ceiling. Most serologists would say that 2 or 3 mg./ml. is a good response, and this is the sort of thing you would aim to get eventually, whatever antigen you are using to immunize with. Those
three quite well established facts of serology are a sort of translation of
the data you presented here, aren't they?

Simonsen: On current hypotheses, I don't see any obvious reason why
the amount of proliferation in the antibody-forming machinery should
be different, between strong and weak.

Mitchison: You may be working up to a ceiling. A rabbit can only
make, say 10 mg. of antibody per ml. You may do well to begin with
but there is less room for expansion subsequently.

Simonsen: I find it hard to believe that it is all due to increased re-
activity of cells which do not multiply. I think it is well established,
particularly in classical immunology, that hyperimmunization is
accompanied by proliferation of antibody-forming cells.

I will gladly accept the analogy you draw to classical antigens which
elicit their maximum response with different rapidity, some quickly and
others slowly. But this fact is itself in need of an explanation. Who
knows, perhaps this has also to do with heterogeneity in the population
of immunologically competent cells, similar to the one I have postulated.
Also the antigens you are mentioning might be classifiable in terms of
how big a fraction of the cell population they have affinity to, when
they are first injected. Perhaps there are also obligatory antigens among
the classical ones, whilst others are more or less facultative.

Michie: Another possibility which would also fit in with the facts is
that with strong histocompatibility antigens in the mouse, you have
already got pre-formed immunity; this could also give the same
correlation that you've shown us, couldn't it?

Simonsen: I think that pre-formed immunity, as far as circulating
antibodies can be taken as a reflection of it, is ruled out. Dr. Amos
would know that better than I. Have any pre-formed haemagglutinins
in the H-2 system ever been demonstrated?

Amos: No. I think there seems to be some sort of non-specific
immunity, especially perhaps in the F_1, but I don't think there has ever
been any convincing demonstration of haemagglutinating antibody.

Michie: Has anybody induced splenomegaly with haemagglutinating
antibodies pre-formed or otherwise? Because if not, I am not quite
sure if this fully meets the possibility of pre-formed immunity.

Simonsen: I am not aware that anyone has done it. I doubt if anyone
has tried it seriously. I think Siskind once tried it, without success.
Medawar: Did not Wigzell try this?

G. Klein: He tried, but I don’t think his results are consistent enough to be able to attribute splenomegaly to humoral antibodies.

Eichwald: You had very large numbers of animals per group. This implies of course that there were several litters per group. How much variation in spleen size was there from litter to litter within the same group?

Simonsen: In my Table I, I showed this as a standard error for each dose level. There is quite a variation from one litter to another in the sensitivity to a given dose but nevertheless when you pool the data from several litters you get a very nice dose-response curve. Of course the variation in sensitivity from one litter to the other would affect the immune cells and the normal cells equally, which makes the variation between the litters rather irrelevant.

Eichwald: A related question: to what extent is it permissible to divide up the litter and mark members of the litter by a surgical or chemical method, either painting them red or green or cutting off the left toe or the right ear, and thereby setting various nutritional or infectious hazards? The mother may not like the altered infant and therefore it does not get a good nipple and as a result you have a small mouse.

Voisin: It should not affect the weight of the spleen.

Simonsen: The mothers always cheerfully accept these marked animals; and I have no indication that it affects the health of the mice. There are always negative controls, that is to say animals injected with isologous cells, and they don’t runt or show other peculiarities compared to non-injected controls.

Medawar: Of course one could randomize the marking system and thus eliminate that source of error, and at the same time make it quite impossible to interpret one’s results!

Michie: If you don’t randomize the marking system, you will, in course of time, find a systematic and spurious difference between two animals in the same litter which ought to show no difference, so that if cutting off a tail has an effect, it will show up.

Brent: J. B. Solomon reckons that there is some sex effect when producing splenic enlargement in chickens, the degree of enlargement depending on the sex of the donor and the recipient. Does this enter into your graft-versus-host assay?
Simonsen: I have never been able to find it in mice.

Hildemann: Is Dr. Simonsen’s hypothesis not really a negation of clonal selection theory, in the sense that all lymphoid cells are supposed to be capable of reacting to strong isoantigens? It seems to me that once you admit that a given lymphoid cell is competent to respond to a number of antigens, then clonal selection loses much of its merit.

Simonsen: I hope I made it clear that the clonal selection in the original version in which Burnet proposed it—that is to say, a heterogeneous population of immunologically competent cells consisting of many small clones each with a different reactivity—seems to me very difficult to fit in with these results; whereas I think they fit well with the modifications I have made of the concepts of Lederberg and Burnet, i.e. that all immunologically competent cells are originally endowed with the same reactivity but some reactivities are maintained better than others, that mutational losses affect certain loci of spontaneous activity more frequently than others, and therefore you do in fact end up with a sort of heterogeneous population where all have to respond to certain antigens, but to other antigens only some would respond.

Medawar: The original Burnet hypothesis of the multiplicity of clones was devised for, and had the merit of, explaining the phenomenon of immunological tolerance, which it does very simply and neatly, but its subsequent modifications and readjustments lose that virtue, it appears to me, without gaining any other virtues in compensation.

Simonsen: I think that the modification I have mentioned gives a plausible explanation for why tolerance is induced with differential ease, but of course it doesn’t contribute at all to the basic question of what tolerance means to the individual cell.

Woodruff: Surely there is one very great gain from a point of view of credibility in Simonsen’s interpretation as distinct from the Burnet “Mark I”, in that it eliminates the Calvinistic element in Burnet, the awful predestination of the cells!

Silvers: Most clonal selection theories of antibody formation are expressed in terms of gene mutation and I do not understand why this is the case. As far as I am aware most biologists don’t explain differentiation in terms of gene mutations. I like to consider it in terms of the segregation of cytoplasmic components which are unequally distri-
buted in the unfertilized egg to begin with—that is, differentiation occurs because the genetic material of one cell does not have the same kinds, or amounts, of substrates to work with as the genetic material of another cell. I wonder why a similar mechanism couldn’t be involved in the establishment of antibody-forming clones?

Simonsen: I would have no objection to that at all.

Loutit: I don’t know whether I have not fully understood your hypothesis, as demonstrated in your Fig. 2. If you invoke mutation, which I always think of as random, why do you get the particular pattern which you illustrated? If it is random there should be $n$ patterns which are possible, yet it seems to me that only that pattern explains your results. It looked to me as though something directed this process rather than random selection.

Simonsen: I wouldn’t like you to take the last diagram too literally. All I meant to illustrate was the idea that the different loci (I am speaking now in the strict mutational sense) determining the spontaneous affinity to different antigens may have different mutability. And if so, it would lead to a heterogeneous population of the kind I have been talking about.

G. Klein: I agree with Prof. Woodruff that this is more attractive than the predestination theory. I would agree also with Dr. Silvers that this is probably more akin to differentiation than mutation, but Dr. Silvers implied that differentiation is due to cytoplasmic segregation. In fact, however, differentiation is due to something about which we know nothing. So I would suggest that we use some more non-committal term than cytoplasmic segregation, or mutation; we could just call it variation.

Silvers: I would go along with that.

Simonsen: I agree too.
FURTHER STUDIES ON INTERACTIONS BETWEEN SESSILE AND HUMORAL ANTIBODIES IN HOMO-GRAFT REACTIONS

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It is now clear that extremist views attributing the breakdown of all allogeneic grafts to a single mechanism can no longer be held. In a specific set of conditions, a particular factor may play a predominant rôle; but the degree of influence exerted by this factor changes with the conditions. For example, Billingham and Sparrow (1954) have shown that the growth of dissociated rabbit epidermal cells may be inhibited by immune serum, whereas the growth of undissociated tissue was not. In mice the influence of cellular factors in the rejection of allogeneic skin grafts becomes less prominent as the host is subjected to further immunization. The cellular infiltration so characteristic of a primary graft is markedly less evident in the “second-set” reaction. Hyper-immunization may lead to a “white graft” type of rejection, which Chutna and Pokorna (1961) consider to be the result of an Arthus phenomenon. It is uncertain whether humoral antibody plays a significant part in the destruction of a primary skin graft. But its function must be of considerable importance in the immune host in view of the histological features of graft rejection in such animals, and the effects of passively administered hyperimmune antisera demonstrated by Stetson and Demopoulos (1958) and

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Chutna and Pokorna (1961). In cases where the transplanted tissue is acutely susceptible to the cytotoxic action of immune serum, such as leukaemias, spleen and lymph node cells, or bone marrow, the relevance of humoral antibody to primary graft destruction is more obvious. But cellular sensitization undoubtedly occurs, and if the experimental conditions are unfavourable to the host, cellular as well as humoral forces may be necessary in order to achieve the rejection of such tissue. Gorer (1958) describing the histology of the breakdown of E.L.4, a C57BL leukaemia, when transplanted subcutaneously to incompatible hosts, states that although extensive graft destruction is visible well before cellular infiltration occurs, invading histiocytes are quite capable of destroying any leukotic cells which remain. Amos (1960) has made similar studies upon both E.L. 4 and L1210 transplanted to the peritoneal cavities of foreign hosts. This site is considerably more propitious for the growth of these tumours, and it is noteworthy that in these circumstances the host cellular response fulfils a more decisive rôle. It seems therefore reasonable to consider these two aspects of a host response to be complementary, and the efficiency of any one particular pattern of response would depend upon the relative proportions of the two elements and the vulnerability of the target at the time and site of exposure.

Theoretically, cellular and humoral factors might act independently of each other, producing an additive effect upon the graft, or they might act synergically. Although the ultimate fate of the entire graft could be the same in both cases, synergic action implies co-operation between components in the destruction of the same target cell. Some experiments have been performed at the late Dr. P. A. Gorer’s laboratory with the purpose of investigating the interrelationships of immune cells and sera. The first of these have been briefly described (Batchelor, Boyse and Gorer, 1960; Batchelor, 1962). Four groups of mice were grafted subcutaneously with a standard suspension of an incompatible ascites
sarcoma, BP 8. The control group received no additional treatment, but the remaining three groups were given either a subeffective dose of isogenic lymph node cells “sensitized” against BP 8, or an amount of host-strain anti-BP 8 immune serum calculated to produce enhancement, or a combination of the “sensitized cells” and serum. In contrast to the other groups, marked inhibition of tumour growth occurred in the animals receiving both the subeffective dose of sensitized cells and serum.

While these results suggested that cells and serum might act in harmony, the experimental system used was not ideal for an exact analysis of the mechanisms involved. The present authors therefore carried out a series of similar experiments in which BP 8 was transplanted intraperitoneally to incompatible recipients. The C3H ascites sarcoma, BP 8, is a suitable tumour for this type of study as it has a distinctive microscopic appearance, and its growth at this site is uncomplicated by plaques of solid neoplasm. Accurate counts of tumour and host cells are therefore possible, and viability can be assessed.

Groups of 6 BALB/c or (BALB/c × C57BL)F1 hosts were given identical intraperitoneal injections of BP 8 in doses between 4 and 6 million cells. Control mice had no further treatment. The groups receiving serum were injected intraperitoneally 2–3 hours before tumour inoculation with BALB/c or (BALB/c × C57BL)F1 anti-BP 8 serum. Doses are indicated in Table I, but in all cases were such as to be incapable of causing suppression of tumour growth if given alone. Enhancement was usually observed. Isogenic sensitized cell suspensions were prepared from lymph nodes draining the site of a subcutaneous implant of BP 8 injected 5 days previously. To obtain the maximum yield of sensitized cells these mice were injected subcutaneously in 6 places and the draining inguinal, axillary and brachial nodes on both sides harvested. The animals inoculated with sensitized cells received them mixed with the tumour suspension. As in the previous experimental design, some mice received serum or sensitized cells
only, and others received a combination of cell and serum treatment. Three mice from each group were killed on the 7th and 9th days respectively after injection of the tumour. The ascitic fluid from each was measured and counts of both tumour and host cells made. The mean cell number of each group was determined, and the differences between groups calculated. Standard errors of the differences between groups confirm the statistical significance of the results.

Unlike the conclusions reached from the experiments in which BP 8 was transplanted subcutaneously, results were obtained demonstrating an antagonistic relationship between sensitized cells and isoantiserum. Some inhibition of tumour growth was produced by one million sensitized cells, and higher doses led to greater suppression. If the cell-treated animals were also given isoantibody, interference with this inhibition resulted. Table I

Table I

ANTAGONISTIC EFFECT OF ANTISERUM UPON THE INHIBITION OF INTRAPERITONEAL GROWTH OF BP 8 CAUSED BY SENSITIZED CELLS

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Serum dose in ml.</th>
<th>Sensitized cells dose in millions</th>
<th>Group mean cell number of BP 8 in millions at -</th>
<th>Difference in millions between group mean and mean of mice receiving sensitized cells only ± S.E. at -</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>363</td>
<td>179 (± 79) 500 (± 47)</td>
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<td>6/3</td>
<td>0.01</td>
<td>2.7</td>
<td>184</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>0</td>
<td>456</td>
<td>* *</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>2.7</td>
<td>501</td>
<td>202 (± 40) 418 (± 119)</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>2.7</td>
<td>386</td>
<td>176 (± 24) 634 (± 114)</td>
</tr>
<tr>
<td>21/3</td>
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<td>0</td>
<td>394</td>
<td>332 (± 36) 403 (± 57)</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>2.2</td>
<td>62</td>
<td>0 0</td>
</tr>
<tr>
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<td>0.005</td>
<td>0</td>
<td>876</td>
<td>* *</td>
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<tr>
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<td>684</td>
<td>* *</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>2.2</td>
<td>286</td>
<td>224 (± 61) 415 (± 57)</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>2.2</td>
<td>300</td>
<td>238 (± 31) 137 (± 16)</td>
</tr>
</tbody>
</table>

* These differences are not included as they are irrelevant to the experimental conclusions.
J. R. Batchelor and M. S. Silverman illustrates two representative experiments in the series. Comparison of the growth of BP 8 in otherwise untreated mice with those which received 2.7 million sensitized cells in experiment 6/3 or 2.2 million in experiment 21/3 shows that inhibition of the tumour was clearly observable by the 7th day and even more obvious by the 9th day. This protection afforded by sensitized cells was abolished by serum treatment. In other experiments, higher cell dosages were injected. It became apparent that the antagonistic action of serum could be overcome at these higher cell dosages, and, inconstantly, figures suggesting synergic action between sensitized cells and humoral antibody were obtained. It will be of great interest if this point can be established with certainty.

The development of the host cellular response was not inhibited by the quantities of serum used in these experiments. There was no significant difference between the host cell counts of the control and serum-treated groups. The ascitic fluid of animals pretreated with sensitized cells and no serum contained fewer host cells as well as the already-mentioned diminished number of tumour cells. This result is only to be expected in view of the smaller volume of ascites in this group. Ascitic fluid removed from the test mice receiving both sensitized cells and isoantisera contained host cells in equal numbers to the untreated controls. These data are in agreement with Gorer's histological observations upon the normal early development of the host response to subcutaneous BP 8 in serum-treated hosts (Gorer, 1958). Since our observations were not carried beyond the 9th day, it is not known whether the host cellular response would have disappeared prematurely. But it was obvious by this time that in the group of mice receiving serum only, despite the presence of large numbers of host reactive cells no effective destructive action on the part of these cells was occurring. By the 9th day in the controls, clumps of dead tumour-host cell aggregates were frequent; in contrast, none were found in the mice treated with serum only.

Since it is uncertain what rôle is played by humoral antibody
in the primary rejection of allogeneic skin grafts, we decided to examine the possibility of synergic activity occurring between humoral and cellular components in this context. To perform such experiments one must know what dose of immune cells is capable of a detectable effect *per se*, and then employ smaller quantities. Attempts were made to titrate sensitized cells by assessing the number needed to produce a well defined adoptive immunity against skin grafts. The experience of Billingham, Brent and Medawar (1954) when demonstrating adoptive immunity in CBA hosts against A skin indicated that at least four immune nodes per recipient would be required; Mitchison (1955) estimated that approximately fifty million sensitized cells were the minimum number able to transfer a detectable level of immunity to Sa in C57BR/a and C57BL hosts in his experiments. Winn (1961) has been able to observe evidence of adoptive immunity to Sa in C57BL/10 hosts with lower doses of sensitized cells also administered intraperitoneally, and in contrast to the other authors found the spleen possessed marked immune activity as early as 7 days after implantation of the immunizing graft. In order to economize on our mouse stocks used for the production of sensitized cells, we injected each animal at 6 subcutaneous sites in relation to the axillary, brachial and inguinal lymph nodes and once intraperitoneally. Five days later the spleen and lymph nodes were harvested, and pooled cell suspensions prepared in “199” medium. Donors of sensitized cells were in all cases of the same strain and sex as the recipients.

Comparisons between sensitized cell dosage experiments performed by different investigators are fraught with difficulties. In addition to the usual strain differences, there are the different test systems of unequal sensitivity. The grafting of H-2 incompatible skin is a sensitive method of revealing immunity, but a less delicate test for showing enhancement. The reverse may be true of some tumour grafts, and marginal degrees of enhancement are best sought for by the use of such material; often it will also be
necessary to employ a particularly favourable strain and sex combination. The duration of antigenic stimulus to which cells are subjected is an important variable, as Mitchison emphasized. He noted evidence of adoptive immunity following transfer of lymph nodes taken 3–5 days after the implantation of the immunizing tumour graft (Sa 1). Billingham, Brent and Medawar (1954) took nodes at 11 days from animals sensitized by allogeneic skin. Although immunity can be detected with regularity after 4 days' application of alien skin (Berrian and McKhann, 1959) when tested by means of further skin grafts on the same host, in our experience the activity of adoptively transferred draining lymph node cells from BALB/c ♂ hosts grafted bilaterally with C3H skin is weak at the 5th day, judged by tumour inhibition but of marked potency by the 11th day. By the 5th day after multiple-site immunization with neoplastic tissue sensitized cells of obvious activity can be obtained. But it is possible that the type of immunological activity may be influenced by this method of sensitization. During this work it has been assumed that such lymph nodes, known to contain sensitized cells but presumed not to be at their peak of humoral antibody synthesis, are the most suitable for synergic experiments. If endogenous humoral antibody synthesis is proceeding at such a rate as to cause a surplus in excess of that absorbed by the graft, further serum injected by the experimentalist is unlikely to provide a factor essential for graft destruction.

The initial experiments were performed on C3H mice grafted with A-strain skin. The hosts were injected intraperitoneally with pooled spleen and lymph node cells sensitized against an A-strain mammary tumour homogenate (AMT 3), and challenged with A-strain skin grafts on the same day. No evidence of adoptive immunity was observed even when 165 million pooled lymph node and spleen cells were administered. The cell-treated animals frequently showed slight prolongation of skin survival times, and it was considered possible that these might represent enhancement.
Similar experiments were therefore performed in which immune-cell-treated and control groups were challenged with AMT 3. Adoptively transferred enhancing activity was likely to be more easily detected in these circumstances. Fig. 1 shows the result of one of these experiments, performed in C3H♂ with 5 animals per group. The three groups of mice treated intraperitoneally with

\[
\begin{align*}
\text{TUMOUR SIZE} \\
\text{DAYS} \\
\end{align*}
\]

![Graph showing subcutaneous growth of AMT 3 in C3H♂ hosts.](image)

\text{Fig. 1. Subcutaneous growth of AMT 3 in C3H♂ hosts.}

- control group
- immune cell group

Fraction denotes number of dead mice/total number in group. Immune cell treated mice received pooled lymph node and spleen cells from isogenic hosts sensitized five days previously with AMT 3. Tumour size is plotted as average diameter in mm. of group.

doses of sensitized cells varying from 104 to 26 millions showed so little difference that they have been charted as a single group of 15 mice in the graph. All animals were injected subcutaneously with aliquots of AMT 3 suspension prepared by mincing the tumour and straining the resultant brei through a sieve. Subsequent tumour growth was measured after depilation of the overlying skin. Two diameters at right angles were recorded and the average diameter in mm. of the group calculated. It can be seen that enhanced
tumour growth occurred in the mice receiving sensitized cells; ultimately 5 out of 15 animals succumbed whereas in the control group all animals survived with no trace of tumour beyond the third week. There is no doubt that AMT 3 grown in C3H ♂ is a tumour-host combination particularly favourable for the demonstration of immunological enhancement, and this must partly account for the observation of adoptively transferred enhancing activity rather than immunity. It is also possible that differences in activity between spleen and lymph node populations might be a subsidiary reason. The pooled immune cell preparations contained a high proportion of spleen cells. Unfortunately the relevant cell counts were not made so that the ratio of spleen cells to lymph node cells is unknown. But further experiments are in progress to test whether any differences exist between the activity of these two populations. At present another tumour-host combination is also being tested for adoptive transfer of enhancing activity. The preliminary experiments have given inconsistent results, but weak adoptive enhancing activity has been observed in some.

Prehn (1959) has reported that splenectomy may have an inhibitory effect on the growth of DBA sarcoma 49 transplanted to BALB/c hosts preimmunized intravenously with DBA blood. While investigating the influence which the route of antigen administration may have upon immunization, he found that 0.001 and 0.002 ml. of defibrinated DBA blood given intravenously to BALB/c mice led to the production of a firm immunity at the 12th day against Sa 49. When the dose of antigen was increased to the equivalent of 0.25 ml. of blood, vigorous tumour growth in excess of that shown by the controls resulted. If, however, these animals were subjected to splenectomy, the progressive growth of the subsequently grafted Sa 49 did not occur, and the mice behaved as if immune. It is difficult to explain these observations except on a basis of immunological enhancement. The possibility of immunological paralysis mentioned seems unlikely in view of the antigen dosage and the
reversion of the test mice to an immune state after splenectomy. If the observed phenomena are connected with immunological enhancement, it must be postulated that under certain conditions of immunization the spleen becomes an organ engaged predominantly in humoral antibody synthesis. Since we found that adoptively transferred enhancing activity could be conveyed by pooled spleen and lymph node suspensions in some test systems,

![Graph](image)

**Fig. 2.** Influence of splenectomy in C3H♂ hosts grafted subcutaneously with AMT 3.

- — splenectomy
- — sham operation

Fraction denotes number of mice still bearing tumours/total number in group. Tumour size is plotted as average diameter in mm. of group.

it seemed of interest to examine in these hosts the effect of splenectomy upon the growth of incompatible tumours. Fig. 2 illustrates the growth curves of AMT 3 transplanted to sham-operated or splenectomized C3H♂. The mice in each group were subjected to identical procedures, short of actual removal of the spleen in the sham-operated group. After one week they were challenged subcutaneously with a suspension of AMT 3 as previously described. Reference to the figure shows that the removal of the spleen had a protective effect upon the host. Fig. 3 illustrates equivalent results obtained in BALB/c ♂ challenged subcutaneously with BP 8 after splenectomy or sham operation. It is
of interest that this effect is more easily obtained in BALB/c ♂ than in ♀, a finding similar to the demonstration of enhancement following passive immunization with isoantibody.

It may be worth while now to recall some of the known facts bearing upon immunological enhancement. Theoretically this phenomenon may result from a modification of the grafted tissue or of the host reaction, or of both. Evidence upon modification of the graft is incomplete. Kaliss (1958) has recorded that enhanced tumours transplanted to further incompatible hosts may retain their characteristic pattern of progressive growth over several transplant generations. Although Snell and co-workers (1960) have recently failed to observe similar behaviour in another test system, the positive results of Kaliss cannot be ignored. It is unknown, however, whether humoral antibody absorbed upon the transferred tumour cells would be sufficient to perpetuate the conditions leading to enhancement. Kaliss (1958) rejected the

Fig. 3. Influence of splenectomy in BALB/c ♂ hosts grafted subcutaneously with BP 8.

--- splenectomy
--- sham operation

Tumour size is plotted as average diameter in mm. of each group of 5 animals. BP 8 inoculum = 5.4 million cells.
proposition that selection of antibody-resistant cells was the basis of enhancement. He found that an inoculum of "enhanced" Sa I injected into C57BL/K hosts was destroyed if such hosts had been preimmunized 7 days previously, but identical preimmunization 14–28 days previously led to yet greater enhancement. Boyse in unpublished work at this laboratory has subjected enhanced mammary tumours, dissociated by an enzymic method, to cytotoxic tests and found that no change in population susceptibility to isoantibody could be associated with enhanced growth.

There are rather more data on modification of the host response. The afferent blocking theory (Snell, 1956; Billingham, Brent and Medawar, 1956) originally proposed that appropriate antigens do not reach the host lymphatic system in sufficient quantity to immunize. In a recent paper (Snell et al., 1960) Snell and co-workers described how passive immunization of B10.D2 hosts grafted with Sa I inhibited the rate of development and the level of immune activity attained by the draining lymph nodes. To explain Kaliss' demonstration that enhancement may result from serum injected up to 10 days after graft implantation (Kaliss, 1958), Snell and colleagues suggest that the maintenance of a high level of cellular hypersensitivity requires a constant supply of antigen. Inhibition of immunization, particularly when following the administration of large doses of antibody as was the case in Snell's experiments, may well be one factor; but it cannot provide a full explanation. Enhancement is one of the most sensitive methods available for the detection of isoantibody, and the small amounts of serum required for its induction do not prevent the initial stages of the host response from occurring. Both Gorer's histological evidence (Gorer, 1958) and our own data show that host reactive cells invade the graft site in normal quantities but their destructive function is incompletely fulfilled. Later they may disappear from the graft site. The results we obtained suggest that sensitized cells are unable to function with
efficiency in an environment containing excess humoral antibody. Humoral antibody synthesis is known to continue during enhanced tumour growth. It must be assumed that some activated cells continue to synthesize and maintain an excess of circulating humoral antibody. This excess has a dual influence, discouraging effective action by cells in a state of specific hypersensitivity and also inhibiting further lymphoid cells from becoming hypersensitive. Our experience has been that the higher the ratio of humoral antibody to sensitized cells, the greater the degree of interference observed. But as toxicity of serum upon its target is directly related to its concentration, the net result represents a balance of these forces.

It is possible that antiserum may directly affect sensitized cells, but a more probable explanation would be that it combines with all the target cell determinants, denying the availability of these sites to act as receptors for the adsorption of sensitized cells. This implies that the adsorption of sensitized cells to their specific target is a reaction very similar to the adsorption of humoral antibody, and Boyden and Sorkin’s conception of cytophilic antibody may be relevant here (Boyden and Sorkin, 1960).

It is well known that lyophilized tissue is capable of immunizing when administered in small doses, but following larger ones, enhanced growth of transplants may be encouraged. It is not easy to reconcile this with any theory of “blocked” immunization, but it could be assumed that this and certain other forms of immunization favour the stimulation of a humoral response at the expense of the hypersensitive cellular form. The view that delayed hypersensitivity reactions tend to be suppressed by high levels of humoral antibody is consistent with data from other fields of immunology. For example, hyperimmunization with the offending antigen is an accepted method of therapy for patients suffering from delayed hypersensitivity reactions to drugs and pollens. In guinea pigs, procedures designed to inhibit the early development of humoral antibody synthesis—such as
immunization by the intradermal route, use of small antigen dose particularly as antigen-antibody complexes (Uhr, Salvin and Pappenheimer, 1957), sublethal irradiation (Salvin and Smith, 1959)—all prolong the delayed hypersensitive state which tends to disappear as humoral antibody synthesis accelerates. Such an explanation might also account for the examples of prolonged graft survival when immunization by the intravenous route with non-paralysing antigen doses has been used (Billingham and Sparrow, 1955; Prehn, 1959). Leskowitz and Waksman (1960) have shown that immunization by this route in rabbits is known to provoke a good humoral response, but a low level of delayed hypersensitivity which is further depressed by a booster intravenous dose. In guinea pigs delayed reactions to chemicals such as 2:4-dinitrochlorobenzene are rarely achieved when subcutaneous and intravenous immunization is performed (Landsteiner and Jacobs, 1936). The inhibition of experimental allergic encephalomyelitis in rabbits, rats, and guinea pigs by prior treatment with nervous tissue unmixed with adjuvants increases with repeated immunization. In a discussion upon this subject Waksman (1959) mentions the possibility that the various forms of delayed hypersensitivity may be diminished by a “vigorous conditioning of the immune apparatus to another type of response”, namely a humoral one.

The adoptive enhancing activity described has been previously observed by Mitchison and Dube (1955). Presumably the inconsistent results obtained in the BALB/c hosts are related to the fine balance existing between the cells secreting humoral antibody, and those in a state of hypersensitivity. The influence of splenectomy has been examined by previous workers (for references see Woglom, 1929) some of whom, as in this paper, record increased resistance. If this represents the prevention of a degree of autoenhancement, it is to be expected that not all tumour-host combinations will allow the observation of this effect, particularly those tumours of extreme sensitivity to isoantisera.
Should the principle be upheld that excess circulating humoral antibody tends to suppress the induction and activity of cells in a state of delayed hypersensitivity, it may be of value in the treatment of some autoimmune diseases. Some authors believe that the action of hypersensitive lymphoid cells plays a major rôle in the tissue destruction. If high titres of humoral antibody are not toxic per se, the suppression of the sensitized cells may be accomplished by this means.

**Summary**

The intraperitoneal growth of the C3H ascites sarcoma, BP 8, in non-immune recipients is inhibited by mixing the tumour inoculum with lymphoid cells derived from isogenic mice previously sensitized against BP 8. Interference by host strain anti-BP 8 isoantibody with the protective effect of immune cells has been demonstrated. Passive immunization of BALB/c and BALB/c × C57BL hosts injected with BP 8 did not inhibit the development of a host cellular reaction; but despite the presence of host reactive cells, graft rejection was not completed. It is concluded that such cells function less effectively in an environment of humoral antibody excess.

Enhancing activity has been transferred by pooled immune spleen and lymph node cells to C3H ♂ hosts. Splenectomy has been found to increase the resistance of C3H ♂'s to an A-strain mammary tumour, and BALB/c hosts to BP 8.

The implications of these findings are discussed in relation to immunological enhancement, and delayed hypersensitivity reactions.

**Acknowledgements**

The authors are deeply indebted to the late Dr. P. A. Gorer F.R.S., on whose ideas they have freely drawn. Thanks are due to Miss A. Carsons and Miss B. Hand for technical assistance and to the Medical Illustrations Department for help with Table I.
REFERENCES


DISCUSSION

Medawar: In some of your earlier experiments on synergistic action, you were dealing with the influence of very small doses of antiserum—0.5 μl. in one case—on the kind of reaction produced by 2.2-2.7 million sensitized cells. Do you know what titres of humoral antibodies are produced merely by the sensitized cells themselves?—because this must
be of the same order of magnitude as the amount of antibody that you are introducing passively. These experiments are almost inevitably confusing, because the cells you are transferring are also probably forming antibodies, aren't they?

Batchelor: This is a difficulty that seems to be almost insurmountable. We try to get around this by taking cells sensitized five days after graft implantation; we know that they are sensitized by this time, from a lot of other evidence, and we know that their maximum antibody synthetic capability has by no means been reached by this time. I quite agree that humoral antibody synthesis by transferred cells of this type ought to be measured but we have not yet done so.

Billingham: I should like to thank Dr. Batchelor for attempting to interpret the prolongations of skin homograft survival that Dr. Elizabeth Sparrow and I obtained in rabbits following intravenous injection with either leucocytes or epidermal cells from the future skin donor. Some years ago Dr. P. Gorer suggested that enhancement might be the basis for this prolongation. Histological examination of biopsy specimens from skin homografts whose survival had been considerably prolonged by this artifice indicated that a reaction frequently did occur at an early stage. Although unable to cause very rapid tissue destruction, it was probably responsible for the inability of the grafts to regenerate hairs. Sometimes in the centres of these grafts the epithelium became weak despite the fact that the outlying epithelium, of migratory origin, remained very robust. Even at the time we believed we were dealing with a homograft response that was only partially suppressed.

Lawrence: In the human being at least, in the transfer of peripheral blood leucocytes or their extracts, one can transfer only delayed allergy, not the capacity for serum antibody formation—even to agamma-globulinaemic subjects—whereas if one uses human lymph node cells one can transfer both the capacity for serum antibody formation and the delayed allergy. I don't know what the situation is in other mammalian species, but perhaps by using leucocytes from the heart blood of the animal it might be possible to dissociate cells that are engaged only in producing delayed allergy and not transferring the capacity for serum antibody.

I would like to make another comment with respect to the rôle of serum antibody in another system. Patterson (1962. In 2nd Inter-
national Symp. Immunopathology, ed. P. Grabar and P. Miescher. Basle: Schwabe) has recently found that in the development of allergic encephalomyelitis (AE) for example, those animals which have re-recovered from “AE” and are producing high titre of circulating serum antibody usually do not come down again, upon challenge with spinal cord, with the disease compared with other animals that produce little or no antibody. To confirm the rôle of serum antibody as a protective agent in this situation, he finds upon transferring high-titre serum antibody to rats actively sensitized to nervous tissue, allergic encephalomyelitis is suppressed or prevented entirely.

Batchelor: I believe it has been reported that patients with agammaglobulinaemia are particularly susceptible to rheumatoid arthritis, and it seems quite possible that this is all connected with the protective or blocking effect of humoral antibody upon delayed hypersensitivity reactions.

Mitchison: Dr. Lawrence, you said that human recipients of transferred delayed allergy made no antibodies. What are your limits of detection?

Lawrence: When one says that no antibodies were found it is understood “within the limits of detection currently available”. The experiments that I had in mind were those where Pappenheimer and I transferred delayed allergy to diphtheria toxoid with leucocyte extracts. Here the method for detection of antitoxin employs one of the most sensitive biological systems available, that is neutralization of toxin in rabbit skin. This biological test detects antitoxin quantitatively, even when present in low concentration (i.e. <0.01 mg. per ml. detectable).

Brent: One observation which we made recently seems to be in conflict with some of the interesting experiments which Dr. Batchelor has told us about. In a non-sensitized animal the sensitizing action of a cell-free antigenic extract can be prevented or counteracted in some way by the injection of antisera. However, if the animal has been presensitized by a relatively small number of allogeneic cells, this counteraction can no longer be brought about. In your experiments you are dealing with an effect of antiserum on presensitized cells, and you are getting exactly the opposite result.

Batchelor: I am not trying to suggest that the results of Snell et al. (1960, loc. cit.) are not genuine, and I am sure that if you put in a lot
of isoantibody you may have some afferent blocking effect; but I think also that antibody is capable of interfering with activated immune cells.

This question of which direction your immune response takes is very peculiar. Why should you get one type of response rather than the other? For example, in the allergic encephalomyelitis story, why should a single inoculation with normal brain tissue unmixed with adjuvant set your immune apparatus in a direction so that you don’t subsequently respond with allergic encephalomyelitis?

**Barrett:** Dr. Batchelor referred to Dr. R. T. Prehn’s work on dosage and route of antigen administration and said that he thought the results should be explained on the basis of immunological enhancement rather than paralysis. I would like to reinforce this. Dr. Prehn and I did many of our experiments with the same materials at the same time. With my methods of immunization, but with the same materials, I have used 20 times that dose and seen only the standard immunity, so I feel confident that that dose is not only not paralysing but it isn’t even close to being paralysing.

Dr. Prehn used a slightly different technique from the one I used, but with the same animals and the same tumours. His inocula were always intracutaneous and it was only under these circumstances that this enhancement was seen. Equal doses of all other materials would give immunity if the transplants were subcutaneous, and the slight differences which occur between Dr. Prehn’s and my work are due to differences in the dosages and differences between the subcutaneous and the intracutaneous sites. This tumour, as has been remarked previously in this meeting, will kill 90 per cent of BALB/c animals when inoculated in small doses subcutaneously, but when inoculated in small doses intracutaneously it will regress in all animals. The balances are quite delicate.

**Feldman:** I am in favour of any theory of enhancement which does not imply a blocking of the afferent direction of antigens. Our experiments on passively-induced enhancement showed that enhanced tumours, while growing progressively in the foreign host, do elicit a high degree of transplantation immunity, which could be demonstrated by passive transfer of the lymph node cells. Several possibilities can be raised to explain this, such as (1) the coating of the tumour cells by humoral antibodies, thus protecting the cells from the cytotoxic effect.
of transplantation immunity, or (2) a direct effect of the antibodies on enhancing the tumour’s growth, thus increasing its resistance to transplantation immunity. It is very hard to distinguish, at this time, between these two possibilities. Dr. A. Globerson and I have attempted, however, to see whether one can distinguish between these possibilities by inducing enhancement in isologous systems. We have tried to enhance tumours with hetero-immune serum in a completely isologous system, and clear indications were obtained that such tumours are enhanced. However, the difficulty is that one can never be sure whether even in these isologous systems there are no immunogenetic differences between the tumour and the host.

Medawar: I don’t quite understand this point, Dr. Feldman, about enhancement in completely isologous systems. What exactly do you do?

Feldman: We took a tumour of strain C57BL, grafted it on mice of the same strain, and then injected antiserum. And the question was will this tumour grow in an enhanced form?

Brent: Prof. Medawar and I no longer believe in the idea that enhancement is due to the coating of the target cells with antibody, so preventing the sensitized cells from reaching the target.

Billingham: I should just like to make one point that may have some bearing on our thinking in this area. Some time ago Dr. Silvers and I undertook a reinvestigation of adoptive transfer of immunity with cells, using A-strain mice tolerant of CBA skin and transferring either “activated” node cells or buffy-coat leucocytes from A-strain mice immunized by means of CBA skin homografts. Eleven days after active immunization of the A-strain mice, 5 million buffy-coat leucocytes obtained from their blood were found to be just as effective in abrogating tolerance as 5 million regional node cells. Even 1 million buffy-coat cells had a demonstrable effect. We wonder whether the cells present in the blood, and which are responsible for transferring immunity, continue to form antibody after transfer.

Mitchison: We can be pretty sure that the peripheral lymphocytes do make antibodies, because Gowans has shown that thoracic duct lymphocytes can transfer the capacity to produce antibody.

Medawar: These are isoantibodies?

Mitchison: They are hetero-agglutinins, I believe.
IMMUNOLOGICAL COMPETENCE OF SMALL LYMPHOCYTES IN THE GRAFT-VERSUS-HOST REACTION IN MICE*

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Since the relatively recent demonstrations of the existence and immunological nature of graft-versus-host reactions in mice (Billingham and Brent, 1957) and chickens (Simonsen, 1957), this subject has been explored so rapidly that a large literature has already accumulated. It is now well known that when adult, immunologically competent cells are injected into non-isologous animals incapable of destroying these cells, a syndrome called runt disease, homologous disease, secondary disease or more generally transplantation disease, usually ensues. The basic immunogenetic and pathological features of transplant-induced disease have been recently reviewed (Billingham, 1959; Tyler, 1960; Oliner, Schwartz and Dameshek, 1961) and need not be detailed here. From a clinical standpoint, reactions produced by grafted cells against their hosts constitute a major barrier to the induction of reciprocal tolerance and the repair of radiation injury. Nevertheless, such reactions have provided a sensitive test system for immunogenetic studies employing inbred lines of animals (Billingham and Brent, 1959; Simonsen and Jensen, 1959; Payne and Jaffe, 1961).

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In nearly all investigations, heterogeneous populations of leucocytes have been transplanted. It has thus been shown that immunologically competent cells are present in the spleen, lymph nodes, bone marrow, thymus, skin, blood—indeed, probably in all adult tissues where leucocytes are prevalent. Following Simonsen's (1957) demonstration that adult cells capable of inciting transplantation disease propagated in chick embryos, Terasaki (1959) attempted to identify the cell type responsible. This study revealed that blood lymphocytes from adults regularly produce graft-versus-host reactions in chick embryos, whereas monocytes have no detectable effect. Taken together, the findings of Simonsen and of Terasaki implicated the lymphocytic system as the source of the mischief-making cells in birds. With this background and the knowledge that about 80 per cent of the leucocytes in mouse peripheral blood are lymphocytes (Dunn, 1954), we proceeded to develop a method for obtaining such lymphocytes in pure preparations for testing in the graft-versus-host assay. Our efforts received additional impetus when Anderson, Delorme and Woodruff (1960) and Billingham and co-workers (1960) discovered that injection of homologous thoracic duct lymphocytes regularly causes runt disease in newborn rats. Interest was then centred on whether large and medium lymphocytes capable of division or small lymphocytes presumed to be incapable of mitosis were effective. We were also intrigued by the challenge that no definitive function had ever been ascribed to small lymphocytes (cf. Perry et al., 1959).

The objectives of the present study were (1) to obtain pure preparations of mouse lymphocytes and assess the competence of these cells in the adult graft-versus-neonatal host system; (2) to evaluate the transplantation reactions produced in relation to such factors as cell dosage, age of recipients at time of injection, pathology and median survival time; (3) to ascertain whether these lymphocyte preparations contained cells capable of further replication and prolonged survival in neonatal hosts.
Materials and methods

Separation of lymphocytes from the granulocytes and monocytes of mouse blood was not easily accomplished. Initially we tried the procedures of Jago (1956) and Cassen, Hitt and Hays (1958) as applied to human blood, and a number of modifications, including the method Terasaki (1959) successfully employed with chicken blood. None of these techniques yielded concentrated preparations of mouse lymphocytes with a high degree of purity and viability. After much experimentation, the following procedure proved efficacious. Adult mice were lightly anaesthetized with ether and blood was collected from a pocket cut between the skin and thoracic wall after severing the subclavian artery. About 7–8 ml. blood was mixed with 0·01 ml. of heparin sodium (1,000 U.S.P. units/ml.) in a calibrated centrifuge tube. This blood was filtered through a glass wool column previously prepared as follows:

Wash Pyrex glass wool with hot running water, rinse with distilled water and dry. Make a small hole in the bottom of a Wassermann tube; pack 1·5 to 1·8 cm. of glass wool in the bottom of the tube as tightly as possible and autoclave. This setup is for C57BL blood. Different lines of mice require different amounts of glass wool for optimum yield and purity of lymphocytes.

The glass wool removed most, but not all, of the granulocytes and monocytes from the blood which was collected in a sterile Wassermann tube. This preparation was mixed and centrifuged at 60 g for 10 minutes, followed by 500 g for 15 minutes. A long Pasteur pipette with a narrowed orifice (sufficient to let a small rubber bulb fill with air in 2–3 seconds after it has been squeezed) was used and the buffy coat was carefully removed, care being taken not to aspirate more than the uppermost layer of red cells along with the lymphocytes. The above manipulations should be performed at 20–25° C; good separations were not achieved at
low temperatures. From an initial 7 ml. of heparinized blood, 0.5–0.8 ml. of lymphocyte-erythrocyte suspension, containing about 1 million lymphocytes/0.05 ml., was obtained. The preparation may be concentrated up to about 1.5 million lymphocytes/0.05 ml. by centrifugation and removal of plasma, but preparations too heavy with cells were not well tolerated by newborn mice following intracardiac injection. With certain strains of mice, red cell contamination was greatly reduced by sedimentation from the filtered blood with polyvinylpyrrolidone according to the method of Walford (1960). Alsever’s solution rather than heparin as anticoagulant gave better separation when polyvinylpyrrolidone was used. Unfortunately, for unknown reasons, several modifications of the latter procedure did not work well with C57BL/6 blood. We were not able to obtain good yields of viable mouse lymphocytes by various dextran-sedimentation procedures (cf. Skoog and Beck, 1956; Walford, 1960). However, we have recently obtained pure and concentrated small lymphocyte preparations after sedimentation of red cells from filtered blood by treatment with fibrinogen (Skoog and Beck, 1956).

Lymphocyte counts of purified preparations were made in Neubauer chambers in the usual manner. For differential counts, Wright-stained smears were prepared and thoroughly examined for contamination with leucocytes other than lymphocytes. Erythrocyte contamination was not considered, since donor erythrocytes were assumed to be immunologically null in the graft-versus-host assay. Cell viability was assessed by eosin dye exclusion (Hanks and Wallace, 1958) employing 1 per cent Eosin-Y. Final preparations were 96–100 per cent pure and 99–100 per cent viable with respect to small lymphocytes. After some experience with the procedure, many preparations were obtained that showed no leucocytes other than small lymphocytes among hundreds of cells scored. The purity is directly, and the yield of lymphocytes inversely, proportional to the amount of glass wool employed in filtration.
Our choice of the strain combination C57BL/6 adult cells→A/Jax newborns or C57BL/A F₁ newborns was based on the finding by Billingham and Brent (1959) that C57→A was one of the few combinations that led to 100 per cent deaths from runt disease when 4–10 million C57 spleen cells were injected intravenously. However, it is probable that neither of their sublines was isogenic with our C57BL/6 (H-2ᵇ) and A(H-2ᵃ) lines obtained from the Roscoe B. Jackson Laboratory, Bar Harbor.

Newborn mice were injected by the intracardiac route using the ingenious technique devised by Grazer (1958). This approach has the advantage of prompt systemic dispersion of the inoculated cells. No magnification is required and there are no post-injection haematomas. The newborn mouse will easily tolerate the injection of 0·05 ml. of cell suspension and there is usually no leakage after withdrawal of the 30-gauge needle.

To assess the mitotic potentialities of the peripheral blood lymphocyte preparations, the tritiated thymidine-radioautographic techniques of Bond and co-workers (1958) and Gavosto, Maraini and Pileri (1960) were employed. Tritiated thymidine was added to cells suspended in plasma-Hank’s solution (1 : 1) in amounts of 0·25 µC to 1·0 µC per 10⁷ cells per millilitre and incubated at 37° with gentle agitation. Samples were removed at intervals up to three hours, centrifuged to concentrate the cells, and smears were then made. The smears were fixed either in Carnoy’s solution, or in methanol followed by IN-HCl to remove any acid-soluble radioactivity. Slides were washed well with tap water, and stripping film (Kodak Ltd., London, AR 10 fine-grain film) was applied in a humid atmosphere. Slides were incubated in the dark for periods ranging from one week to six weeks. The film was then developed and the slides stained with Wright’s stain.

The pattern of development of transplantation disease in neonatal mice was studied quantitatively by the weight-gain assay of Russell (1960) and by the liver-spleen enlargement assay of
Fig. 1. Purified preparation of lymphocytes and red cells from adult C57BL/6 blood. Photograph of Wright-stained, air-dried film. No leucocytes other than small lymphocytes were detected in many preparations capable of producing lethal runt disease in newborn A/Jax mice.
Simonsen and co-workers (1958). To test for acquired tolerance, single, full-thickness skin homografts were made from adult C57BL/6 donors, employing the technique of Billingham and Medawar (1951).

Results

Characterization of the lymphocyte preparations inoculated

As indicated in the previous section, the final preparations injected into neonatal mice were 96–100 per cent pure and 99–100 per cent viable with respect to lymphocytes. Since the problem of lymphocyte purity was of paramount importance in these studies, high cell yield was sacrificed to obtain pure preparations. With many of the preparations injected, no monocytes or granulocytes whatever were observed among hundreds of lymphocytes counted on Wright-stained films. Nevertheless, not all of the lymphocytes could confidently be classified as small lymphocytes on the basis of morphology alone (see Fig. 1). It was, therefore, crucial to determine whether large or medium lymphocytes, known to be capable of mitosis, were present in the preparations injected.

Several preparations of purified, peripheral blood lymphocytes from C57BL/6 adults of both sexes were tested by the tritiated thymidine-radioautographic technique previously described. The film strips subsequently developed, after incubation of the slides for periods ranging from one week to six weeks, revealed no uptake of labelled thymidine whatever among thousands of lymphocytes counted. Hence, the supposition that our preparations contained no appreciable number of blood cells capable of mitosis was confirmed, and the assumption that we were employing small, and not medium or large, lymphocytes was reinforced. It might still be argued that these small lymphocytes, assumed to be end cells, may divide after localization in certain tissues outside
the blood. However, all the evidence from the present series of studies is against this possibility.

**Transplantation disease as a function of cell dosage**

Our initial experiments were directed toward ascertaining the dosage of C57BL/6 peripheral blood lymphocytes required to procure runt disease in newborn A/Jax mice. These results are summarized in Table I. Lymphocyte doses of 200,000 and 750,000 cells failed to induce any discernible graft-versus-host reactions. The injected animals developed quite normally. When 800,000–900,000 cells were inoculated by the intracardiac route on the day of birth, however, symptoms of runt disease were subsequently apparent in 12/19 animals with two deaths ensuing. Further experiments revealed that about 800,000 cells constituted a threshold dose of small lymphocytes for the induction of runt disease in this strain combination. Injections of 0.9–1.5 million small lymphocytes produced a high incidence of lethal transplantation disease. Over this dosage range, there was no substantial difference in the severity of the disease syndrome. Ninety-six of a total of 111 injected mice were affected with symptoms varying from failure to gain weight at the normal rate, with eventual recovery, to death following profound systemic disease. In this group 66/111 animals or 59.5 per cent succumbed. The time distribution of deaths in this series is plotted in Fig. 2. It will be noted that the earliest death occurred at nine days and only one animal survived beyond 30 days. This represents a normal time-mortality distribution with a median survival time (MST) of 16.4 (15.4–17.5) days within 95 per cent confidence limits. If the data are considered as a truncated distribution with \( n = 111 \), taking into account also the animals which failed to die within 70 days, a median survival time of 21.2 (19.5–23.2) days is obtained. For reasons to be considered below, the MST of 16.4 days is probably a better quantitative approximation. Animals dying or cannibalized within five days after injection were
## Table I

**Induction of runt disease in neonatal A/Jax mice following intracardiac injection of adult C57BL/6 small lymphocytes**

<table>
<thead>
<tr>
<th>Lymphocyte dose per mouse</th>
<th>Age at injection (hr.)</th>
<th>No. litters</th>
<th>Initial no. experimental survivors†</th>
<th>No. runt disease deaths</th>
<th>Runt disease evident</th>
<th>No gross pathological changes evident</th>
<th>Median survival time in days, with 95% confidence limits in parentheses</th>
</tr>
</thead>
<tbody>
<tr>
<td>200,000</td>
<td>&lt; 8</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>750,000</td>
<td>&lt; 20</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>800,000–900,000</td>
<td>&lt; 20</td>
<td>5</td>
<td>19</td>
<td>2</td>
<td>10</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>900,000–1 million</td>
<td>&lt; 24</td>
<td>10</td>
<td>47</td>
<td>27</td>
<td>13</td>
<td>7</td>
<td>16.4 (15.4–17.5) as normal distribution; 21.2 (19.5–23.2) as truncated distribution with n = 111</td>
</tr>
<tr>
<td>1.0–1.2 million</td>
<td>&lt; 24</td>
<td>10</td>
<td>41</td>
<td>27</td>
<td>10</td>
<td>4</td>
<td>17.4 (15.6–19.6) as normal distribution; 19.9 (17.8–22.3) as truncated distribution with n = 45.</td>
</tr>
<tr>
<td>1.3–1.5 million</td>
<td>&lt; 24</td>
<td>6</td>
<td>23</td>
<td>12</td>
<td>7</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>2 million</td>
<td>&lt; 20</td>
<td>13</td>
<td>45</td>
<td>35</td>
<td>5</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>2.5 million</td>
<td>&lt; 24</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>1 million</td>
<td>&gt; 24 &lt; 48</td>
<td>3</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>900,000§</td>
<td>&lt; 24</td>
<td>4</td>
<td>18</td>
<td>14</td>
<td>4</td>
<td>0</td>
<td>10.8 (9.2–12.7) as normal distribution; 13.1 (10.5–16.4) as truncated distribution with n = 18</td>
</tr>
</tbody>
</table>

* One or two mice in each litter left uninjected as controls with tail tips cut off; all showed normal growth and survival.
† Animals dying or cannibalized within 5 days after injection are considered to be technical losses and are not included in the results.
‡ Survivors were test-grafted at 6–10 weeks of age with C57BL/6 skin; none showed acquired tolerance.
§ Small lymphocytes harvested from C57BL/6 mice immunized by two i.p. injections each of cells equivalent to 1/4 adult A/Jax spleen 6 and 13 days previously.
considered to be technical losses and are not included in the results.

When newborn A/Jax mice each received two million adult C57BL/6 small lymphocytes, 35/45 or 77.9 per cent died of runt disease, whereas 40/45 or 89 per cent gave evidence of graft-versus-host reactions. Although the incidence of runt disease was substantially greater at this higher cell dosage, the time-mortality distribution revealed no acceleration of the syndrome. Thus an MST of 17.4 (15.6-19.6) days was calculated for the normal distribution, whereas an MST of 19.9 (17.8-22.3) days was obtained on the assumption of a truncated distribution with $n = 45$. Technical difficulties in the isolation of viable blood lymphocytes in higher dosage have not yet been surmounted. More highly concentrated preparations than those obtained by the straightforward filtration-centrifugation procedure described contained so many red cells that immediate or very early death resulted when newborns were injected by the intracardiac route.

Fig. 2. The time distribution of deaths of 63 A/Jax mice that received 0.9-1.5 million C57BL/6 small lymphocytes via the heart during the day of birth.
However, in one litter all animals which were successfully injected with 2.5 million lymphocytes died of typical runt disease. In all of these experiments, one or two mice in each litter were left as uninjectected controls identified by cutting off the tail tips; these animals showed normal growth and survival.

Following the injection of one million lymphocytes by the intracardiac route into neonatal mice >24 <48 hours old, none of the mice showed evidence of runt disease and all developed normally. Thus rapid maturation of the A/Jax host’s immune response capacity within two days after birth apparently led to the elimination of homologous donor lymphocytes before any visible damage was done.

Peripheral blood lymphocytes from specifically preimmunized C57BL/6 mice were tested to evaluate further the immunological competence of these cells on a quantitative basis. Small lymphocytes were harvested as in earlier experiments from adult C57BL/6 mice immunized by two intraperitoneal injections each of cells equivalent in amount to \( \frac{1}{4} \) adult A/Jax spleen at 13 and 6 days previously. A/Jax mice from four litters less than 24 hours old were injected with about 900,000 “immune” lymphocytes—equivalent to a dose of normal lymphocytes just sufficient to procure runt disease. To avoid immediate reactions that could be attributed to preformed donor serum antibodies, the cells were washed repeatedly in cold Hank’s solution prior to injection. All 18 recipients were severely affected and 14/18 died of acute runt disease between seven and 20 days of age. The MST’s of 10.8 (9.2–12.7) days or 13.1 (10.5–16.4) days on the assumption of a truncated distribution clearly reveal that sensitized lymphocytes are capable of mediating accelerated transplantation disease (cf. Table I). The efficacy of such cells even in relatively low dosage is also attested by the finding that no animal escaped immunological attack.

The apparent absence of pathological changes in certain animals under conditions where the majority of animals receiving the same
cell inoculum died of acute runt disease was at first difficult to reconcile with the well-defined immunogenetic system employed. However, a scrutiny of the technical variables involved pointed to inadequate cell dosage as a probable factor. This could be attributed to (1) failure to remix the cell preparation frequently, with consequent settling of cells during the course of injections, and (2) occasional leakage of a small drop of the inoculum from the heart puncture after withdrawal of the needle. From this standpoint, the median survival times calculated on the supposition of truncated distributions are less valid than those based on death of all animals receiving 900,000 or more lymphocytes. Nevertheless, the same qualitative conclusions may be drawn on either basis.

**Evaluation of lymphocyte-induced transplantation disease by the weight-gain and organ-enlargement assays**

All animals employed in this investigation were maintained under standardized conditions recommended for optimum growth and development (Byerly, 1957). Neonatal mice from many litters were individually weighed from the 5th to the 48th day of age. Weight-gain curves were then determined for normal A/Jax mice and for A/Jax mice that received 0.9–1.5 million C57BL/6 small lymphocytes via the heart during the day of birth. For the most part, the normal mice were littermates of the injected mice. Mean weights with their standard errors are plotted in Fig. 3 for 64 normal mice and for 107 experimental mice, representing a total of 433 and 542 individual weights, respectively. The curve best fitted to the normal weight-gain values reveals a sigmoid distribution that levels off after 30 days and rises rapidly again after 40 days. The levelling is probably a consequence of weaning at 30 days and adjustment to solid food. A significant curtailment of mean weight increase reflecting runt disease in experimental animals was evident at nine days, which was also the day the first death occurred. From this time until about 30 days, the disparity in weight gain between experimental
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and control animals remained conspicuous. Although the majority of experimental mice died of acute runt disease at two to

![Graph showing weight-gain curves for normal A/Jax mice and for A/Jax mice that received 0.9-1.5 million adult C57BL/6 small lymphocytes via the heart during the day of birth. Mean weights with standard errors are given for 64 normal mice and for 107 experimental mice, representing a total of 433 and 542 individual weights, respectively.]

The weight-gain assay continues to reveal the effects of graft-against-host reactions in survivors up to 35 days of age (cf. Fig. 3). Thus the weight-gain
assay provides a satisfactory basis for an overall measurement of varying degrees of runt disease over a broad range of ages.

The general predictive value of the weight-gain assay employed was borne out by individual weights in the series that received $2 \times 10^6$ cells. All animals that later died showed individual weights well below normal from 9–11 days on, although many eventual survivors achieved weights approaching or within the normal range by three weeks of age.

A more detailed but laborious assay of graft-induced transplantation disease, first devised by Simonsen and co-workers (1958), involves the determination of body, spleen and liver weight indices of graft reaction. The method is based on the finding that with many combinations of inbred mouse strains, there is an increase of spleen and liver weights in conjunction with failure to gain body weight among neonatal mice under immunological attack. This approach, of course, requires that injected litters be killed at a given time, optimally at some 9–15 days after injection. Evaluation of histopathological changes during the syndrome was also made concurrently in the present study.

Application of this “organ-enlargement” assay to our system provided insight into the immunopathological effects of blood lymphocytes compared with those of the mixed populations of leucocytes employed in previous mouse studies. Four litters of A/Jax mice totalling 12 experimental animals were killed at 14 and 15 days of age. These animals had been injected by the intracardiac route with $0.9–1.0$ million adult C57BL/6 small lymphocytes at birth. Whole body weights were taken, gross pathological changes noted, and spleen, liver and kidney weights recorded for each animal prior to fixation of these organs for subsequent sectioning and staining with Maximow’s haematoxylin-eosin azure II stain (spleens) or haematoxylin and eosin. From these data, indices of graft-versus-host reaction were computed along with the means and confidence limits for each set of values. These results are detailed in Table II. The indices are quotients represent-
### Table II

**Indices of graft-versus-host reaction at 14–15 days after injection of $0.9 - 1.0 \times 10^6$ adult C57BL/6 small lymphocytes into A/Jax newborns**

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Age in days</th>
<th>Chief gross pathological features</th>
<th>Whole body indices</th>
<th>Mean ± s.e. (s.d.)</th>
<th>Spleen indices</th>
<th>Mean ± s.e. (s.d.)</th>
<th>Liver indices</th>
<th>Mean ± s.e. (s.d.)</th>
<th>Kidney indices</th>
<th>Mean ± s.e. (s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>Caseous and pale liver</td>
<td>0.595</td>
<td>1.88</td>
<td>2.06</td>
<td>1.27</td>
<td>1.12</td>
<td>0.997</td>
<td>1.16 ± 0.05</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>Slightly caseous liver</td>
<td>0.822</td>
<td>1.88</td>
<td>1.68</td>
<td>1.12</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>Normal</td>
<td>0.990 ± 0.09</td>
<td>1.31</td>
<td>1.57 ± 0.12</td>
<td>1.14</td>
<td>1.64 ± 0.13</td>
<td>0.997</td>
<td>1.16 ± 0.05</td>
<td>1.14</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>Caseous and pale liver</td>
<td>0.834</td>
<td>1.55</td>
<td>1.62</td>
<td>1.10</td>
<td>1.31</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>Caseous liver, severe diarrhoea</td>
<td>0.424</td>
<td>1.24</td>
<td>1.70</td>
<td>1.31</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>Caseous and pale liver</td>
<td>0.779</td>
<td>1.52</td>
<td>1.33</td>
<td>1.15</td>
<td>1.17</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.17</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>Pale but non-caseous liver</td>
<td>0.621</td>
<td>1.27</td>
<td>1.23</td>
<td>1.17</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>Caseous and pale liver</td>
<td>0.667 ± 0.06</td>
<td>1.31</td>
<td>1.30 ± 0.12</td>
<td>0.977</td>
<td>1.30 ± 0.08</td>
<td>1.00</td>
<td>1.19 ± 0.06</td>
<td>1.19</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>Normal</td>
<td>0.835</td>
<td>1.29</td>
<td>1.42</td>
<td>1.22</td>
<td>1.31</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.19</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>Caseous and pale liver</td>
<td>0.989</td>
<td>0.943</td>
<td>1.11</td>
<td>1.11</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>Pale spleen</td>
<td>0.790</td>
<td>1.34</td>
<td>1.28</td>
<td>1.14</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.14</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>Nearly moribund, caseous and pale liver, very pale spleen</td>
<td>0.415</td>
<td>0.480</td>
<td>1.72</td>
<td>1.56</td>
<td>1.10</td>
<td>0.907</td>
<td>1.16 ± 0.05</td>
<td>1.10</td>
</tr>
</tbody>
</table>

* Proportion of significant indices italicized above: 5/12 2/2 3/12 1/2 5/12 2/2 3/12 2/2

* Indices determined according to method of Simonsen et al. (1958). Individual indices more than two standard deviations (s.d.) from 1 are italicized as significant; likewise, mean indices more than two standard errors (s.e.) from 1 may be regarded as significant.
ing the body weight or relative organ weight per 100 grams body weight over the mean of the respective weights of two littermate controls. Thus indices greater than 1 indicate organ enlargement, while indices less than 1 indicate a body or organ weight less than that of the control animals. Statistically significant differences are evidenced by individual indices more than two standard deviations from 1; likewise, a mean index value more than two standard errors from 1 may also be regarded as significant.

Three animals showed no gross pathological changes at autopsy. The weight indices of these mice similarly indicated no significant deviation from the normal. The remaining nine animals all revealed pathological changes, most consistently in the liver as evidenced by anaemia and/or focal necrosis. However, grossly normal lymph nodes were observed in all animals. High liver indices (enlargement) were closely associated with severe runting (low body weight indices) and conspicuous pathological changes. Significant kidney enlargement occurred only in conjunction with severe runting, which suggests that kidney failure is involved in the latter stages of the fatal disease. Although the body weight indices of all 12 animals were less than 1, only five individual indices were significantly depressed. Nevertheless, the mean indices for both 14 and 15-day groups were significantly low. Inspection of the liver and kidney indices similarly revealed significant organ enlargement which was, however, much greater for the liver. Although marked splenomegaly was also observed, atrophy of this organ was pronounced in animal No. 12, which was nearly moribund at the time of assay. For this reason, only one of the two mean spleen indices is significantly high. Heart weight indices were also determined and none differed substantially from the normal. It may be noted that liver and spleen indices were high in two animals (Nos. 2 and 4) that showed only slight body weight depression, whereas the reverse situation obtained with animals 7 and 8. From an overall quantitative standpoint, the mean body weight and liver indices most sensi-
tively reflect the occurrence of graft-versus-host reactions in this system.

Histopathological changes in tissue sections from these animals may be summarized as follows. The white pulp follicles of the spleens of normal A/Jax mice are compact with definite perifollicular "collars", but the follicles of runts were more diffuse and hypertrophied with indistinct perifollicular "collars". Moreover, the red pulp of runts showed far more pale-staining and swollen reticuloendothelial cells as well as accentuated erythropoiesis. In contrast with littermate controls, enlarged livers of runts showed oedema and focal necrosis with vacuolation and loss of cytoplasm in hepatic cells. In other areas, coalescence of cells with indistinct cytoplasmic membranes was observed. No marked changes were seen in sections of enlarged kidneys from runted animals.

**Non-tolerant and non-chimeric state of inoculated survivors**

Most of the experimental animals that failed to die of acute transplantation disease (cf. Table I) were test-grafted with C57BL/6 female skin at six to ten weeks of age. Littermate controls were similarly test-grafted at the same time. All of these homografts were rejected between 8 and 12 days after grafting, indicating that none of the A/Jax mice neonatally injected with C57BL/6 small lymphocytes had been made tolerant of C57BL/6 antigens. The absence of accelerated reactions to these homografts also indicated that the experimental A/Jax mice had acquired no lasting immunity as a consequence of neonatal exposure to C57BL/6 lymphocytes. In another approach to the question of tolerance or persistence of donor cell descendants, an attempt was made to renew the runting syndrome in animals that had recovered from the initial graft-versus-host reaction. Eleven such survivors about 12 weeks old that had not been tested with skin homografts were each injected intravenously with 3.4 million adult C57BL/6 blood lymphocytes. None showed any signs of
distress or disease following the injection. These animals were also weighed regularly for a month after injection and all showed normal weight gains.

A more direct test for chimerism in A/Jax survivors of runt disease was made by the cytotoxic isoantibody method devised by Gorer and Boyse (1959). Briefly, potent A anti-C\textsubscript{57} and C\textsubscript{57} anti-A isoimmune sera were obtained following four intraperitoneal injections totalling about 300 million leucocytes (lymph node, spleen, and bone marrow) per recipient. Each test tube contained $0.1\text{ ml.}$ of antiserum dilution ($1:4-1:64$), $0.1\text{ ml.}$ of 1 per cent lymph node cell suspension, and $0.05\text{ ml.}$ of guinea pig complement previously absorbed with mouse cells at $0\degree C$ to remove naturally-occurring antibodies. Appropriate serum and complement control tubes were included. After 60–90 minutes' incubation at $30\degree$, $0.05\text{ ml.}$ of 1 per cent Eosin-Y was added to each tube; 200 or more cells from each test mixture were then scored and the proportion of stained cells determined. All dilutions were made with Medium 199 (Hyland Laboratories), since preliminary tests indicated that cell viability persisted for many hours in this medium. A total of 14 A/Jax survivors of the runting syndrome was tested by the above method. In all instances, a high proportion of the lymph node cells was stained by Eosin-Y after reaction with C\textsubscript{57} anti-A serum, although there was no evidence of cytotoxic reactions after exposure to A anti-C\textsubscript{57} serum. Typically, 60–80 per cent of the test cells were stained in the presence of anti-A/Jax serum, and 10–15 per cent were stained after incubation with anti-C\textsubscript{57} serum. This low percentage of stained cells in the latter tests was also observed in the serum and complement control tubes. Some cell death is unavoidable as a result of teasing the cells from the nodes. The potency of the A anti-C\textsubscript{57} serum was verified by its high cytotoxicity for C\textsubscript{57}BL/6 lymph node cells under identical conditions. These experiments then clearly support the assumption that no substantial number of C\textsubscript{57}BL/6 lymphoid cells persisted in A/Jax mice that recovered
from transplantation disease. Since death of all or none of the test cells was not obtainable, it is possible that a very low proportion of donor type cells might have been present, but not detected.

**Tolerance without disease mediated by foetal liver cells**

The finding that A/Jax survivors of runt disease were neither tolerant of C57BL/6 cells nor chimeras with respect to such cells indicates that the originally inoculated lymphocytes failed to leave descendants. Before these results could be further evaluated, it was necessary to establish whether A/Jax newborns could be made tolerant of C57BL/6 tissue under any circumstances.

Several litters of A/Jax mice less than 24 hours old were injected via the heart with foetal C57BL/6 liver cells. Each animal received 1.3 million viable nucleated cells. As expected, none of these mice subsequently showed any signs of graft-versus-host reactions. At eight weeks of age, 12 of these animals were test-grafted with adult female C57BL/6 skin. All recipients were found to be tolerant and most were highly tolerant with homografts surviving in excellent condition for 60 days or more. Thus the failure of adult lymphocytes to induce tolerance must be attributed to the characteristics of these cells, rather than to the nature of the host or the antigenic disparity in this strain combination.

**Transplantation disease in F₁ (C57BL/6 × A/Jax) hybrids**

Early in the course of this investigation, reciprocal intrastrain skin grafts were exchanged between 12 mice of like sexes in both our A/Jax and C57BL/6 colonies. All intrastrain grafts were successful and remained in perfect condition indefinitely. This finding confirmed the supposition that each colony was highly inbred and essentially isogenic with respect to histocompatibility antigens. From a theoretical standpoint, F₁ hybrids from any two such highly inbred parental lines should be more favourable hosts for evaluation of the potentialities of adult parent-line grafts, since incompatibility is possible in only one direction. This
immunogenetic design has, of course, been employed to advantage in a number of recent studies.

We sought to ascertain whether purified preparations of adult C57BL/6 small lymphocytes would produce runt disease in neonatal F1 (C57BL/6 x A/Jax) hybrids similar to that observed in A/Jax newborns. A total of 12 F1 hybrid litters was injected with C57BL/6 blood lymphocytes in doses ranging from 1.25–1.86 million viable cells per recipient. The procedures employed were the same as in previous experiments. The inoculated mice ranged in age from less than 18 hours to four days of age. Three entire litters of mice injected with the same preparation, as well as control littermates, died nine to ten days later with an acute enteric infection, possibly transmitted by a blood donor. Systemic manifestations of disease in these animals at the time of death were so profound that the occurrence of runt disease \textit{per se} could not be established. No special problems were encountered with the remaining litters. In this group there were 48 experimental survivors initially; these animals were regularly observed and weighed beginning seven days after injection.

Much to our surprise, only four mice subsequently died of runt disease. Eight additional animals showed definite evidence of transplantation disease with slight runting within 32 days after injection. Thus 36 of 48 injected F1 hybrids revealed no symptoms of graft-versus-host reactions. It is noteworthy that severe reactions occurred only in F1 hybrids that were less than 48 hours old at the time of injection with C57BL/6 lymphocytes. A clue to the apparent resistance of the F1 animals was their hybrid vigour; normal F1 controls as well as many experimental animals showed weight gains substantially greater than normal A/Jax mice of the same age from seven days on.

\textbf{Discussion}

Although Russell (1960) and others have found that as few as 50,000 adult spleen cells may regularly cause fatal runt disease
in homologous newborns, it is apparent that 800,000 or more small lymphocytes are required to produce the same reactions. The most likely explanation for this difference is that spleen and lymph nodes contain many cells that multiply in the host, whereas the small lymphocytes derived from adult blood fail to do so. On the basis that each immunologically competent cell injected will cause a certain quantum of damage to the neonatal host, one might suppose that death would be hastened at progressively higher cell doses. This was not found over the dosage range tested with lymphocytes from unimmunized donors. However, a moderate dose of 900,000 small lymphocytes from animals specifically presensitized to host antigens did result in a significant acceleration of the runting syndrome with early death. This finding strongly supports the contention that the syndrome mediated by small lymphocytes has a primary immunological basis.

The capacity of isologous, adult A/Jax lymphocytes to protect newborns injected with C57BL/6 lymphocytes has not yet been tested. Such protection with respect to spleen cells in this strain combination has been demonstrated by Siskind, Leonard and Thomas (1960).

A median survival time of $14.9 (13.7-16.4)$ days was calculated from Russell's (1960) data on the time distribution of deaths of 39 C57BL/6 mice that received one to two million DBA/1 spleen cells intravenously during the day of birth. Although the strain combination was different from ours and the ultimate dose of cells probably very high, the MST determined is essentially the same as we found (cf. Table I). Thus it may be inferred that the time course of fatal transplantation disease is much the same whenever (1) a threshold dose of immunologically competent cells is exceeded in newborn, homologous hosts and (2) strong antigens exist in host cells that are lacking in the donor. Even in F1 (C57 × A) hybrids inoculated at three weeks of age with very high doses of C57 or A spleen and lymph node cells, Trentin
(1958) found mean survival times of 16 and 21 days, respectively. Although Billingham and co-workers (1960) give no median or mean survival times for neonatal rats inoculated with various types of homologous, adult lymphoid cells, the range of ages at death suggests that the time course of runt disease in this species is quite similar to that observed in mice. Our early dosage experiments suggested that A/Jax recipients less than eight hours old were more susceptible to severe immunological attack than those 

> 8 < 24 hours old. This supposition was not upheld by later work. Apparent variations in susceptibility of whole litters probably reflect differences in the length of the gestation period and consequent extent of immunological maturation at birth.

The weight-gain and organ-enlargement assays each have distinct advantages. The former permits long-term observation, compilation of survival time data, and tests for tolerance and chimerism; the latter facilitates determination of detailed pathological changes as a function of age and time after injection, although a large number of experimental animals may be required to permit sampling at various ages.

Although high mean liver indices and low mean body weight indices most sensitively reflected the severity of runt disease in our system, Simonsen and co-workers (1958) generally found spleen indices higher than liver indices and only slightly depressed body weight indices in a variety of strain combinations. However, their data are based on F₁ hybrid recipients inoculated with parental spleen cells at ages ranging from 0–14 days. Moreover, they noted that liver enlargement was the more pronounced the younger the host. Other pathological features observed in our experiments are for the most part consistent with the findings of others (Billingham and Brent, 1959; Kaplan and Rosston, 1959; Siskind, Leonard and Thomas, 1960; Russell, 1960; Oliner, Schwartz and Dameshek, 1961) who employed mixed populations of donor leucocytes. The more profound changes observed by Gorer and Boyse (1959) in the spleen and liver of F₁ (C57BL × A)
hybrids inoculated with spleen cells from isoimmune A-strain donors may be related either to the use of very large doses of immune donor cells, the adult status of the recipients, or both. A more detailed account of the histopathological changes induced by small lymphocytes will be reported elsewhere.

The chief difference in our interpretation of graft-versus-host reactions is that host cell multiplication is held responsible for the organ enlargement and accentuated splenic erythropoiesis, whereas the pathological lesions result from the immune reactivity of small donor lymphocytes with a long lifespan. On this premise, the proliferation and intervention of descendants of donor cells are not required for the development of the runting syndrome. That blood lymphocytes normally survive for several or many weeks, rather than for just a few hours or days as previously supposed, has been shown in various recent studies (Hamilton, 1958; Trowell, 1958; Gowans, 1959).

The acquisition of lasting tolerance by A/Jax newborns inoculated with foetal C57BL/6 liver cells without concomitant transplantation disease demonstrates that tolerance does not depend upon widespread destruction of host lymphoid tissue. Conversely, the absence of tolerance and chimerism in A/Jax survivors of runt disease mediated by adult C57BL/6 small lymphocytes supports the assumption that these lymphocytes are end cells that leave no descendants in the host. One could also attribute the failure of immunologically active C57BL cells to persist in A/Jax recipients to injury in a highly foreign antigenic environment (Boyse, 1959) or to exhaustive sensitization (Simonsen, 1960). However, any transplanted cells or their descendants that lacked immunological reactivity should have survived indefinitely in compatible hosts. Earlier evidence that runt disease requires host tolerance, but that tolerance of the graft does not depend upon trauma to the host, has been summarized by Billingham (1958).

The finding that 75 per cent of F₁ (C57 × A) hybrids, inoculated
at 0–4 days of age with A/Jax lymphocytes, showed no evidence whatever of graft-versus-host reactions was unexpected. The apparent hybrid vigour of the hosts and failure of the injected cells to multiply and thus intensify the attack may account for this result. Experiments employing higher doses of donor lymphocytes are now in progress. In this connexion, Trentin’s (1958) observation that lymphoid tissue from C57 parents had only a slight effect on 21-day-old (C57 × A) F1 hybrids, whereas a comparable dose of A-strain cells induced profound disease, suggests that the particular strain combination involved may be important. Contrary to simple immunogenetic expectations, the age of F1 recipients receiving parent strain lymphoid cells has turned out to be an important variable. In our experiments, severe runt disease was induced by small lymphocytes only in recipients less than 48 hours old at the time of injection. With another strain combination, Simonsen and Jensen (1959) found that liver and spleen enlargement regularly occurred only in those F1 animals which were less than 11 days old at the time of injection with 10⁷ parent strain spleen cells. Similarly, Kaplan and Rosston (1959) determined that very young (one to ten day-old) F1 hybrid recipients were more sensitive than adults, but that disease occurred in adults after injection of massive doses of parental strain lymphoid cells (see also Cole and Ellis, 1958). However, Trentin (1958) found that parental strain bone marrow cells in high dosage failed to cause weight loss or death in young adult F1 hybrids. Considering the available evidence as a whole, it appears that relative to host age or degree of development, much higher doses of parental lymphoid cells may be required to cause deleterious changes in young adult or adult F1 hybrids. Hybrid vigour is perhaps responsible for the increased resistance of older animals. Further investigation should reveal the basis for these anomalous results.

The donor cell preparations employed in these studies contained no leucocytes other than blood lymphocytes. These lymphocytes
appeared and behaved quite like small lymphocytes incapable of mitosis. None of the evidence obtained, ranging from dosage experiments to tests for chimerism and attempted tritiated-thymidine labelling, suggests that a significant number of cells capable of multiplication was present among the injected cells. A recent investigation by Cole and Garver (1961) demonstrates that lymphocytes, but not granulocytes, are capable of procuring transplantation disease in mice. However, they attribute the immunological reactivity to proliferating large and medium lymphocytes, and not to small lymphocytes. Although our data clearly indicate that small lymphocytes are immunologically competent cells, it remains to be determined whether these are the only mischief-making cells when mixed populations of lymphoid cells are injected. In apparent conflict with the results of Siskind, Leonard and Thomas (1960), both Simonsen (1960) and Dineen (1961) have found that chimeric spleen cells from runted mice usually fail to produce further runt disease on passage to new hosts. Although the latter workers have interpreted their findings in terms of acquisition of tolerance by adult graft cells, these divergent results might all be attributable to gradual death of the original donor lymphocytes present and/or their failure to multiply.

The finding that small lymphocytes are immunologically reactive, but apparently produce no descendants, has an obvious bearing on clonal selection theories of immunity. Recent work essentially proves the developmental sequence of lymphocytes to be large→medium→small (Everett et al., 1960). Most investigators now agree that large and medium lymphocytes are actively mitotic, whereas small lymphocytes (except thymocytes) are end cells that rarely or never divide (see Ciba Found. Symp. Haemopoiesis, 1960). The notion that small lymphocytes are effete is belied by their content of highly active RNA as well as by their long lifespan. Although there is evidence that some lymphocytes can, under certain conditions, turn into monocytes...
or macrophages, evidence that they can differentiate into any other cell type is unconvincing (Trowell, 1958). Our present results with small lymphocytes are most readily accommodated to the older view that an immunologically reactive cell is capable of coping with diverse antigens. To maintain a clonal selection view of the immune function of small lymphocytes, one must argue that numerous cells of each relevant clone are present in the initial inoculum and respond directly to antigenic stimulation without need for replication of specifically competent cells. However, mitotically active lymphoid cells would participate in immune responses under normal conditions, and such cells are required to account for anamnestic responses in general. In the light of increasing evidence for the existence of lymphocyte-associated cellular immunity as distinct from antibody globulin production (Snell, Winn and Kandutsch, 1961), we are inclined not to regard the capacity to elaborate humoral antibodies as an essential criterion of immune reactivity at the cellular level (see Burnet, 1959). Whether small lymphocytes are capable of dual immune responses is not yet clear.

A recent study of the order of appearance of blood cells in newly hatched and young bullfrog larvae (Hildemann and Haas, 1962) indicates that all types of definitive leucocytes other than small lymphocytes appear during the period when larvae can still be made completely tolerant to homografts. Small lymphocytes were observed to appear and increase about ten-fold in number during the time of transition from the homograft tolerance to immune type of response at 40–50 days post-hatching. In other vertebrates, including man, small blood lymphocytes first appear in late embryonic or early postnatal life. Thus the apparent relationship between the ontogeny of the lymphocytic system and the maturation of the isoimmune response capacity provides additional evidence that small (mature) lymphocytes play an important rôle in transplantation immunity.

Tyler (1960) has lately proposed that spontaneous cancer arises
from single cells that suffer loss or inactivation of histocompatibility genes. Such cells are assumed to react against normal cells possessing the relevant antigen(s) as in experimental graft-versus-host reactions. The precancer cell is supposed to respond to the proliferative stimulus of the new foreign, "host" antigen with the result that cancer cells gradually destroy and replace normal ones. If this hypothesis has general validity, one would expect that animals which escape lethal transplantation disease should later show a high incidence of tumours. We found no evidence of cancer in our runt disease survivors, but these animals were not kept sufficiently long to make negative results convincing in this connexion. The idea of a proliferative stimulus to "graft" cells by "host" antigens is of course in accord with clonal selection theory. The present results merely argue against the necessity for such cell proliferation as a requirement for transplantation disease.

Summary

Purified preparations of small lymphocytes derived from adult C57BL/6 blood were found capable of producing transplantation disease in newborn A/Jax and in very young F1 (C57BL × A) hybrids. About 900,000 small lymphocytes constituted a threshold dose for the induction of fatal disease. Over the dosage range of 0.9–2.0 million lymphocytes, there was no substantial difference in the severity of the disease syndrome. The median survival time for A/Jax mice after injection of 0.9–1.5 million and 2 million small lymphocytes was 16.4 (15.4–17.5) days and 17.4 (15.6–19.6) days, respectively. However, injection of lymphocytes from preimmunized donors led to accelerated transplantation disease with a median survival time of only 10.8 (9.2–12.7) days. This finding strongly supports the contention that the lymphocyte-mediated syndrome has a primary immunological basis.
Both weight-gain and organ-enlargement assays were employed for quantitative evaluations of small-lymphocyte-induced disease. Curtailment of mean weight increases as well as low mean body weight and high liver indices sensitively reflected pathological changes associated with graft-versus-host reactions. The organ enlargement is attributed to host cell multiplication and reaction to immunological attack.

Radioautography of lymphocyte preparations following exposure to tritiated thymidine showed no labelling whatsoever. A/Jax survivors of runt disease were not chimeras, but possessed only A/Jax cells as evidenced by cytotoxic isoantibody tests. Moreover, animals that recovered from runt disease were not tolerant of C57BL/6 skin homografts. Conversely, A/Jax newborns inoculated with foetal C57BL/6 liver cells showed no runt disease, but became highly tolerant of adult C57BL/6 skin homografts. It is concluded that small lymphocytes from adult mouse blood are immunologically reactive, though non-proliferating cells.

Acknowledgements

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REFERENCES

IMMUNOLOGICAL COMPETENCE OF SMALL LYMPHOCYTES

DISCUSSION

Miller: J. L. Gowans, B. M. Gesner and D. D. McGregor (1961. Ciba Found. Study Gp 10, Biological Activity of the Leucocyte, p. 32. London: Churchill) have evidence that suggests that the small lymphocyte can initiate a graft-versus-host reaction. It is true that small lymphocytes cannot be labelled by a single injection of tritiated thymidine, but if you give repeated injections or a continuous infusion of the radioactive label to a rat, a larger proportion of small lymphocytes become labelled. If such labelled cells are injected into an $F_1$ hybrid, then 24 hours after injection, labelled small lymphocytes appear in the splenic white pulp. But in addition there are other types of cells which show labelling in their nuclei: some of these are in mitosis, others have a thin rim of pyroninophilic cytoplasm, a pale nucleus and a single nucleolus, and others are larger, with more abundant, densely pyroninophilic cytoplasm and one or two prominent nucleoli. Is it possible that the small lymphocyte, in order to initiate a graft-versus-host reaction, has to transform into another type of cell such as the pyroninophilic cells described by Gowans and his colleagues? Have you any evidence of such a transformation in your work?

Hildemann: The most recent evidence of which I was aware is contained in the Ciba Foundation volume on Haemopoiesis of last year. Everett et al. (cited in my paper) have demonstrated that the development sequence of lymphocytes is large to medium to small, and that the labelling that one may detect in small lymphocytes is attributable to label that is carried over in the process of division, in going from medium to small. Many investigators have of course argued that small lymphocytes are capable of transforming into a variety of cells. My understanding of the current consensus is that small lymphocytes may under certain circumstances transform into macrophages or monocytes; evidence that they transform into any other type of cell is controversial.

Medawar: I think everyone would be convinced by Gowans' latest evidence, just cited by Dr. Miller, on the transformation of thoracic duct lymphocytes when they are in the course of mounting a graft-against-host reaction. A clear picture is building up of the lymphocyte as a cell which does not proliferate as such because it is really the larval
form of a cell; it has to turn into something different before it engages in immunological responses. As Miller indicated, it settles down and acquires a pyroninophilic cytoplasm, and that is the business or operative form of the lymphocyte. I don’t think this in any way affects any of your conclusions.

Hildemann: I am still concerned about the complete absence of tolerance and chimerism in the survivors and in addition the threshold dose of small lymphocytes required to produce runt disease, if in fact the majority of these cells proliferate in their hosts. Our results are perhaps consistent with the assumption that some small lymphocytes localize in certain tissues outside the blood and there proliferate for a limited time.

Medawar: Dr. Hildemann, your test of chimerism was cytotoxic assay. This would cease to be valid if the cells you are trying to identify didn’t turn up in cell suspensions: if for example they turned into rather sticky or fragile cells that just didn’t come out into a cell suspension.

Hildemann: These were lymph-node cell suspensions.

Medawar: Maybe these transformed lymphocytes simply don’t tease out and don’t become isolated cells which you can identify. The failure to produce tolerance surely follows: if these animals have recovered from runt disease, doesn’t it follow that they are not tolerant—that it was the ones which died that were tolerant?

Loutit: I go along with Dr. Hildemann most of the way—I can concede that these small lymphocytes do not persist indefinitely—but I would follow Gowans and Trowell and say that they can transform and go through a number of cycles which would be enough to produce the graft-versus-host reaction or the runting syndrome. Then, as you say, either death occurs or the cells run out of their potential for division. The whole thing is quite compatible with several views, but it needs further experimental work.

Eichwald: Should we not also consider that the addition of phytohaemagglutinin to peripheral blood stimulates mitotic division—but mitotic division of which cells? Certainly not of neutrophilic leucocytes. Therefore they would be most probably lymphocytes, would they not?

Russell: Gorer and Boyse (1959. Immunology, 2, 182), in their morphological studies of the transfer of parental cells to adult F₁ hybrids,
observed quite a striking population of macrophages, large pale cells, particularly in the spleen, but I think also elsewhere, and they theorized that these might be the principal operative cells. Have you seen these in your animals? We see them rather regularly in our runts following neonatal injections of spleen cells, which means a mixture of different morphological types, of course.

_Hildemann:_ We have not thoroughly evaluated our histological sections, but we see no abundance of such cells in the spleen, nor in the liver.

_Michie:_ I was most interested in Dr. Hildemann’s account of the weight-loss assay and look forward to its further development by Dr. Hildemann and Dr. Russell. There are many purposes for which you don’t want to sacrifice your test animals and this is one potential assay system where we can get round this disagreeable necessity. I have one question about the magnitude of body weight depression which you showed in your Fig. 3. In one of your slides you had a photograph of a couple of littermate mice, where one was _in extremis_ and weighed about 5 g., whereas the other would weigh about 20 g.; differences of that order are commonly encountered in the terminal stages, but the magnitude shown on your graph seems to be of a much more modest order.

_Hildemann:_ I am sorry, that was misleading. The mouse I showed was an exceptional runt and a normal littermate at 48 days of age. All other severely affected runts died within 30 days after injection. I think the reason for lack of greater disparity between the control and the experimental values is simply that the worst affected animals die in great numbers around two weeks of age. The experimental curve gradually comes back up to meet the control curve at about 35 days as the animals with sub-lethal disease recover. That is why I emphasize the point that beyond 30 days in this system, the weight-gain assay no longer distinguishes the experimental and control groups. The experimental curve does not reflect weight loss, but rather the lack of weight gain compared with the controls. The control curve was based on some 500 normal mice including all of the littermate controls in the many litters employed.

_Eichwald:_ In your organ-enlargement assay, could not your raised liver index and kidney index be due to a preservation of the normal
organ weight in an animal whose other tissues have lost weight? Therefore it would not be comparable to splenic enlargement.

_Hildemann:_ The indices are relative to the organ weights of littermate controls. The enlargement of some of the organs is terrific; it is really remarkable that an animal can have such a low body weight and still have such very large organ weights. We have two opposing factors here.

_Eichwald:_ But the organ is not enlarged; the rest of the body is decreased in size.

_Hildemann:_ No, I disagree. We compare each organ with the same organ of littermate controls, or the body weight with the body weight of littersmates.

_Eichwald:_ Per 10 g.?

_Hildemann:_ The calculated indices are quotients representing the body weight or relative organ weight per 100 g. body weight over the mean of the respective weights of two littermate controls.

_Woodruff:_ There is an important element in your argument that you mentioned briefly that I’d like more details about: you said that you got high tolerance easily with foetal liver. I think this is very crucial because until you came to that point in your paper, it seemed to me that the failure to get tolerance could be explained as a peculiarity of the C57 mouse as donor or, in more general terms, as Dr. Simonsen mentioned, that it is difficult to produce tolerance with a combination of donor and host which is commonly associated with severe runt disease. This hypothesis seemed to be borne out by some experiments which Dr. Michie and I performed some years ago in which we tried, but completely failed, to make CBA mice tolerant of C57BL homografts by injecting (CBA × C57) F_1 spleen cells in the neonatal period. I would be interested to know therefore how strong your evidence is that it is easy to produce tolerance with C57BL foetal liver.

_Silvers:_ How many cells did you use?

_Hildemann:_ We got prolonged but not permanent tolerance with as few as 1.5 million viable, nucleated foetal liver cells, and the tolerance was based on survival of female C57BL/6 skin homografts for 60 days or more. We got no acute rejections whatsoever and the majority of grafts survived beyond 80 days.

_Michie:_ As a rider to the experiment that Prof. Woodruff has mentioned, although we couldn’t induce any tolerance with 10 or more
million cells of F₁ hybrid spleen, doses of less than 5 million C₅₇BL spleen cells were sufficient to give a practically 100 per cent kill. This seems to suggest that you can get lethal running under circumstances where you have by no means full, or even detectable tolerance, in this particular system.

Nakić: I would like to protest against the use of the term “liver cells” when what is meant is liver parenchyma cells. Liver cell suspension contains many immunologically competent cells, but I do not believe that tolerance can be induced with liver parenchyma cells.

Hildemann: The point here is that foetal liver cells will become tolerant of the newborn host and vice versa, so you have reciprocal tolerance. This approach has been much used by radiation immunogeneticists in the repair of radiation injury. It is true that foetal liver is a heterogeneous population of cells, and we did this to establish the point that it is possible to make newborn A-lines tolerant of C₅₇ cells in general, but apparently not with C₅₇ small lymphocytes derived from peripheral blood—at least over the dosage range that we have thus far tested.

Brent: I certainly would strongly support the idea that A-line mice can be made tolerant of C₅₇ antigens.Billingham and I found that if newborn A-line mice were injected with roughly 5 million bone marrow cells it was possible to induce tolerance to C₅₇ skin grafts.

Hildemann: We retested this finding because our lines are probably not isogenic with yours, so it was desirable to establish the point once again.

Brent: The point I would like to make is that in my very limited experience of cytotoxic tests, which I carried out with the aid of such experts as Dr. Batchelor and Dr. Silverman, it doesn’t seem to be a very good technique for detecting rather small numbers of cells. In my own experiments the number of donor cells in the chimeras is relatively small; it might be something of the order of 1 to 5 per cent. I doubt that the cytotoxic test can reveal the presence of such a low proportion of cells. It might be more useful to try Mitchison’s test for donor cells—to see whether there are enough donor cells to sensitize normal mice against skin grafts from the donor strain. Such a test can be made to be reasonably quantitative. It might also be helpful to skin graft all your animals in order to reveal how many of them have become tolerant.
**Hildemann:** We had no tolerant survivors at all. I should add that we had no evidence of immunity in these animals either; thus the A-line recipients were not immunized as a consequence of their exposure to C57BL small lymphocytes and subsequent recovery from runt disease. The median survival times were right in the middle of the usual range.

**Billingham:** Dr. Silvers and I have been using the CBA to A mouse strain combination to compare the ability of cells of different histological origins to confer tolerance when inoculated intravenously into neonatal hosts. As might be expected, cell suspensions prepared from nodes and spleens caused runt disease in many of the subjects, with variable mortality. Nearly all the animals which survived the injection of 1 or 2 million node cells proved to be tolerant of CBA skin. Homologous marrow and spleen cells provided much less effective tolerance-conferring stimuli than node cells, though they caused less runt disease.

To eliminate the occurrence of runt disease, (CBA × A) F₁ cells were employed. To our considerable surprise F₁ hybrid axillary and brachial node cells proved to be a highly inefficient tolerance-conferring stimulus—inferior in fact to adult thymus cells. Similar findings have been obtained in experiments with rats. One possible explanation for the wide disparity in results obtained with F₁ hybrid node cells on the one hand and with parental-strain node cells on the other is that the latter are stimulated to proliferate and increase their dosage after inoculation by the antigenic stimulus of their hosts. Some evidence in support of this interpretation comes from the fact that increasing the dosage of F₁ lymphoid cells in rats to a relatively high level does result in the induction of tolerance of subsequent skin homografts.

**Hildemann:** If the small lymphocytes that we are injecting are capable of transforming to other cells in large numbers, namely pyroninophilic cells or “haemocytoblasts”, then why should these A-line mice not have become tolerant of C57 antigens? If, on the other hand, these cells are mostly end cells (which our tritiated thymidine evidence indicates), then the results would stand as I have interpreted them. I agree with Dr. Brent that a small proportion of C57 cells might have been present but not detected in the A-line survivors. The reason for this is that in teasing the cells from the lymph nodes you always damage some, so the cell control tubes (lacking antiserum) always show a low proportion of stained cells. The number of stained cells from A-line
runt survivors in the presence of hyperimmune anti-A serum is at best 80 to 90 per cent. Nevertheless, despite the lack of all-or-none results, the evidence from cytotoxic isoantibody tests does not support the assumption that a substantial number of donor lymphocytes are replicating and leaving descendants.

Simonsen: I too have seen Gowans' evidence and I have been very much impressed by it. But I think we also should quote here the evidence of A. J. S. Davies and S. M. A. Doak (1960. Nature (Lond.), 187, 610) with the chromosome marker technique, which shows that virtually none of the cells in mitosis, in the enlarged spleen, are of donor origin. As far as the mouse goes, we would probably all agree with you that the organ enlargement is mostly or wholly due to a host-cell proliferation. What is really mysterious is not the origin, but the rôle of the pyroninophilic cell, which is proliferating at a tremendous rate in these organs, sometimes constituting more than 50 per cent of the entire cell population. Some of them, as indicated by Gowans' findings, must be of donor origin, but the vast majority of the pyroninophilic cells, if we can accept Davies and Doak's results as being valid for mice in general and not only for their own strains, must be of host origin, and nobody knows what they are doing.
HOMOGR AFT SENSITIVITY IN HUMAN BEINGS*

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In addition to its fascination as a biological riddle, the homograft reaction and its inverse, acquired tolerance, have provided totally new insight into the nature of the normal immune response and its aberration, autoimmune disease (Medawar, 1956, 1958, 1959).

At the practical level any efforts designed to ameliorate or suspend this immunologically specific acquired antagonism of the host toward another's tissues will be greatly facilitated by the precise definition of the antigen or antigens that induce this sensitivity and the antibody or antibodies that function as the instruments of the tissue damage observed (Lawrence, 1956, 1959, 1960a). The opening papers of this symposium have dealt with the considerable recent progress made in the difficult problem of identification and characterization of tissue antigens (p. 6, 45, 72). The present discussion will concern itself with certain biological and immunological properties of a mechanism of homograft rejection that offers the nearest resemblance to an

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antibody-like reagent currently available for analysis of the delayed tuberculin-type of hypersensitivity.

Discussion of mechanisms of homograft rejection are helped by a definition of the exact type of homograft under consideration. Gorer’s (1956) summary of available evidence makes it unwise, if not impossible, to expect the same mechanism to be operative in the destruction of all type of homografts. We will limit ourselves to the response evoked by the orthotopic skin homograft—an organized tissue characterized by the establishment of vascular and lymphatic connexions with the host. This is in contrast to responses evoked by leukotic cells or by ascites tumour cells.

**Transfer factor and delayed bacterial sensitivity**

In order to place the transfer of homograft sensitivity in human beings in perspective, it is appropriate to consider briefly the events leading up to this extension of the biological properties of transfer factor. As an outgrowth of Chase’s (1945) observation using viable cells in animals, there has been found in extracts of human leucocytes obtained from donors with delayed tuberculin-type hypersensitivity a factor (or factors) which when injected into a non-sensitive recipient causes him to respond as the donor to the specific delayed hypersensitivity transferred. The altered reactivity produced in the recipient by this means is prompt, widespread and enduring. The agent involved in the transfer of immunological information has been termed “transfer factor”, with the realization that one or more factors may be involved in the transaction (Lawrence, 1960b). Extracts of human peripheral blood leucocytes containing transfer factor have served to transfer delayed-type hypersensitivity to a variety of bacterial (tuberculin, streptococcal (Lawrence, 1955), diphtheria toxoid (Lawrence and Pappenheimer, 1956)) and fungal (coccidioidin (Rapaport et al., 1960a)) antigens. Confirmation and extension of the findings on the efficacy of non-living extracts of leucocytes
and the transfer of delayed sensitivity of the tuberculin type in human subjects has begun to appear from several laboratories (Freedman, Fisher and Cooke, 1957; Maurer, 1961; Barnett and Sanford, personal communication, 1959; Eisen, personal communication, 1961.) Some of the properties of transfer factor, in so far as they are currently known, have been set forth in an earlier

Table I*  

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<tr>
<th>Biological</th>
<th>Biochemical TF unaffected by:</th>
<th>Immunological</th>
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<tbody>
<tr>
<td>Endows recipient with specific sensitivity of donor</td>
<td>25° or 37°—6 hr.</td>
<td>Interacts with but is not neutralized by antigen</td>
</tr>
<tr>
<td>Sensitivity is systemic</td>
<td>Distilled water lysis</td>
<td>White blood cells (WBC) desensitized by antigen</td>
</tr>
<tr>
<td>Onset early (hours).</td>
<td>Freeze–thaw 10 cycles</td>
<td>Neg. WBC+ antigen→no transfer</td>
</tr>
<tr>
<td>Duration long (months–year)</td>
<td>Deep freeze—5 months effective</td>
<td>No detectable antibody (AB) in donor WBC extract</td>
</tr>
<tr>
<td>Minute dosage WBC</td>
<td>A S little as 0.01 ml.–local transfer</td>
<td>No detectable AB in skin or serum of recipient at time of maximum transferred sensitivity</td>
</tr>
<tr>
<td>As little as 0.1 ml.–systemic transfer</td>
<td>Capacity for transfer depends on degree donor sensitivity and dosage WBC used</td>
<td>DNAse</td>
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<td>RNase</td>
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<td>RNAse</td>
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<td>Repeated test with antigen may increase intensity and duration of transferred sensitivity—yet is not necessarily its cause</td>
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<tr>
<td>Negative donors incapable</td>
<td>Extracts or cell-free supernatants as effective as viable cells</td>
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<td>Does not cross species barrier</td>
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* Reprinted from Lawrence (1960b).
Ciba Foundation Symposium (Lawrence, 1960b) and are summarized in Table I.

Transfer factor and skin homograft sensitivity

The experimental design and the precise conditions ensuring successful transfer of skin homograft sensitivity by means of DNAsate-treated leucocyte extracts obtained from the peripheral blood of specifically sensitized humans have been set out in considerable detail elsewhere (Lawrence et al., 1960). For the purposes of this discussion the basic experimental design followed is: (1) Skin grafts from subject A were used to sensitize subject B. (2) Leucocyte extracts containing anti-A transfer factor were treated with DNAsase and injected into subject C. (3) Sensitivity transferred to C was measured by the survival time of a test graft from A compared to that of a control graft from D. (4) A positive result was scored when the test graft was rejected in an accelerated fashion (4–6 days) while the control graft was accorded a first-set survival time (8–12 days).

In the sensitization and testing procedures full-thickness, 11 mm.-diameter skin grafts were used. The time of rejection was determined by stereomicroscopic and gross criteria for graft rejection described elsewhere (Converse and Rapaport, 1956). The appearance of rouleaux formation in the erythrocytes coursing through the graft vessels, followed by thrombosis, haemorrhage and extravasation of blood, heralded the downfall of the graft. This was later reflected in the gross events of ecchymoses and oedema which progressed to necrosis and eschar formation of the grafted skin.

The isolation, extraction and DNAsase treatment of leucocytes used for transfer of homograft sensitivity followed methods previously described (Lawrence, 1955). The donors of skin and leucocytes fulfilled the rigid criterion of not having transmitted hepatitis following blood donations in the recent and remote past.
Results of local and systemic transfer of homograft sensitivity

Two techniques of transfer were employed:
(i) Local transfer whereby a test and a control graft are placed on each forearm of a non-sensitive subject. On the third day of graft residence, when assurance of vascularization of the grafts is evident, leucocyte extracts obtained from a donor sensitized to the test graft are injected halo fashion 10 mm. away from and around both test and control graft on one forearm, and serum (1/0 ml.) from the same sensitized donor is injected around both test and control graft on the other forearm.

This manoeuvre resulted in accelerated rejection of the test graft (4–5 days) within 24 to 48 hours following transfer although the control graft exhibited no evidence of sickness or death until the eighth day when it was accorded a first-set reaction of rejection. The grafts on the opposite arm of the same recipient treated with serum were both accorded a first-set reaction (test graft survival 10 days, control graft survival 8 days).

Three other recipients bearing only one test and one control graft responded similarly following local transfer (recipient No. 6: test graft survival 4 days, control graft survival 10 days; recipient No. 8: test graft survival 5–6 days, control graft survival 10 days; recipient No. 9: test graft survival 5–6 days, control graft survival 10 days). To control these results further, two additional recipients had two test grafts—one on each forearm. Repeating the above experiment and injecting non-sensitized leucocyte extract or sensitized serum around each test graft had no effect on their survival times—each test graft being accorded a first-set reaction (e.g. recipient No. 7: test graft + sensitized serum, survival time 10 days; test graft + non-sensitized WBC extract, survival time 10 days.)

From the group of experiments it was concluded that although a first-set and second-set skin homograft were insufficient to
sensitize donor blood leucocytes to the degree that is necessary for systemic transfer, this exposure to antigen is sufficient to effect a local transfer of sensitivity. It is known from earlier experience with transfer of delayed sensitivity to bacterial antigens that juxtaposition of antigen and transfer factor increases the intensity of the transfer and allows for a tenfold reduction in dosage of leucocytes (Lawrence, 1949).

It was also concluded that even in this more sensitive transfer system, a positive non-specific effect of non-sensitive leucocyte extracts could not be detected; nor did a specific effect of sensitized donor serum reveal itself.

(2) Systemic transfer: in this technique of transfer the DNAse-treated leucocyte extract obtained from a sensitized donor is injected into the shoulder of the non-sensitive recipient. Eight days later test and control grafts are applied to the recipient's forearm. When this technique is used both grafts become vascularized by the third day of residence. Twenty-four hours after vascularization (on the fourth day) the test graft had undergone the haemorrhage and thrombosis of accelerated rejection while the control graft was accorded a first-set reaction of rejection (e.g. recipient No. 11: test graft survival, 4 days, control graft survival, 11 days; recipient No. 24: test graft survival, 4 days, control graft survival, 13 days).

From this group of experiments it was concluded that although a first-set and second-set exposure to skin grafts was not sufficient to sensitize donor leucocytes to the degree necessary to effect a systemic transfer, a sequential series of four sets of skin grafts applied to the same donor was a sufficient sensitizing antigenic stimulus to allow systemic transfer.

Systemic transfer however will not occur when the donor of leucocyte extract is sensitized by four sequentially applied grafts if each homograft after the first undergoes a "white graft" reaction rather than the usual accelerated reaction of rejection.
The rôle of desensitization

The finding that leucocyte extracts obtained from one donor at the height of his accelerated rejection period of a fourth-set sensitizing skin homograft transferred homograft sensitivity (accelerated rejection) to two recipients is of some interest. Of particular significance, however, was the finding that leucocyte extracts prepared from the same donor 11 days later were incapable of transferring homograft sensitivity to four additional recipients. When the donor of leucocyte extracts was tested with the application of a fifth-set graft of the skin to which he had been sensitized, he accorded it a first-set reaction of rejection. It may be of interest also that the same leucocyte donor exhibited a “recall flare” at each previously rejected graft bed site while in the course of rejecting the second, third and fourth-set grafts (cf. Rapaport and Converse, 1957).

One interpretation of these results could be that the individual actively sensitized by repeated application of skin homografts has his peripheral blood leucocytes only transiently endowed with the capacity to transfer homograft sensitivity. Another, perhaps more likely interpretation, particularly in view of the appearance of the “recall flare” phenomenon, would suggest that in the course of active hypersensitization of the leucocyte donor, the phenomena of sensitization and desensitization proceed pari passu. If this be the case, then when sensitization is in the ascendancy transfer will be successful, and when desensitization has occurred transfer of homograft sensitivity will fail.

There is precedent for this conclusion in the transfer of tuberculin sensitivity in human beings where it has been found that in vitro incubation of sensitized viable leucocytes with antigen (tuberculin-PPD) results in their becoming desensitized and incapable of transferring sensitivity (Lawrence and Pappenheimer, 1957). This observation has been confirmed in vivo in human subjects by showing that leucocytes of donors sensitive to tuberculin
and to streptococcal proteins will transfer sensitivity to both antigens. However, when the same donors are desensitized to tuberculin to the point where the tuberculin skin reaction is lost, their leucocytes also become selectively desensitized to tuberculin and are then capable of transferring sensitivity only to streptococcal proteins (Oliveira-Lima, 1958).

It should be noted in this regard that in all of our own transfers of delayed sensitivity to bacterial and fungal antigens, the leucocyte donor is taken as he occurs in Nature. The transfer of skin homograft sensitivity required a departure from this principle in so far as the leucocyte donor had to be actively sensitized in the period before leucocyte transfer. A summary of the results upon which this discussion has been based is set out in Table II.

Table II

Summary of results of transfer of specific sensitivity to skin homografts*

<table>
<thead>
<tr>
<th>Mode of sensitization and time of collection of WBC from donor</th>
<th>Method of transfer</th>
<th>Material used for transfer</th>
<th>Results of transfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-set rejection</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>2</td>
</tr>
<tr>
<td>Second-set rejection</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>2</td>
</tr>
<tr>
<td>First- and second-set Local rejection</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Local</td>
<td>Non-sensitive WBC extract</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitive WBC extract on control graft</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitive serum on test graft</td>
<td>3</td>
</tr>
<tr>
<td>Fourth-set rejection</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>6</td>
</tr>
<tr>
<td>11 days after fourth-set rejection</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>4</td>
</tr>
<tr>
<td>5 days after fourth-set “white-graft” application</td>
<td>Systemic</td>
<td>Sensitive WBC extract</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sensitive plasma and RBC</td>
<td>1</td>
</tr>
</tbody>
</table>

* Reproduced from Lawrence et al. (1960) with permission from the Journal of Clinical Investigation.
Conclusions

The transfer of homograft sensitivity in human beings confirms the earlier findings of Mitchison (1954) on the transfer of tumour homograft sensitivity in mice; of Billingham, Brent and Medawar (1956) on the transfer of skin homograft sensitivity in mice; and of Brent, Brown and Medawar (1958) on the “transfer reaction” in guinea pigs. The ability of DNAse-treated leucocyte extracts to transfer homograft sensitivity in man is a striking departure from the requirement in animal species for intact, viable cells to achieve this effect. This discrepancy is believed to reflect more the relative ease with which man may acquire and express sensitivity to a variety of foreign materials, rather than any real species difference. It would appear that the difference in the behaviour of the cellular transfer system in homograft reactions arises from the degree of sensitivity possible for various species (mouse, guinea pig, man) to achieve, and the means of immunological expression at the disposal of each (Lawrence, 1960a).

The fact that transfer of homograft sensitivity in humans must be done in genetically diverse outbred populations and the fact that the individual specificity of homograft reactions is just coming under scrutiny (Rapaport et al., 1960b) introduce inescapable variables in evaluating the results achieved. Nevertheless, since the effect of transfer on test grafts has been judged significant only when compared to the behaviour of control grafts on the same recipient, the rôle of such variables, although not fully eliminated, has been greatly diminished.

The unique attributes of the transfer of homograft sensitivity in man which may prove useful in formulating concepts of at least one mechanism of orthotopic homograft rejection are: (1) The demonstrated effectiveness of leucocyte extracts and various cell-free preparations in the transfer of delayed sensitivity, a reality that affords an opportunity for identification and characterization of transfer factor mediating these effects. (2) The
fact that leucocytes or extracts obtained from human peripheral blood have been found incapable of transferring the capacity for detectable serum antibody formation in agammaglobulinaemic subjects (Good et al., 1957) and in normal recipients at the time of maximal transferred delayed sensitivity to diphtheria toxoid, when assayed by a most sensitive means of detecting antibody, even in minute concentration (<0·01 mg./ml.) (Lawrence and Pappenheimer, 1956). (3) The function transferred by leucocyte extracts to human recipients consists of both the prompt effector reagent of delayed allergy (e.g. prompt effects of local transfer of homograft sensitivity) and the machinery necessary for its continued production in the recipient as in the leucocyte donor (e.g. enduring effects of systemic transfer in recipients homografted one to two weeks after injection of leucocyte extracts).

The findings in respect of leucocyte extracts allow application of principles elucidated in relation to delayed sensitivity of bacterial and fungal origin towards an understanding of mechanisms of homograft sensitivity. In this perspective transfer factor may be viewed as a common efferent instrument of tissue damage encountered in the delayed type of altered tissue reactivity whether this event is initiated by bacterial cells, by fungal cells, or by cells of yet another type such as are found in tissue homografts.

**Summary**

The transfer of local or systemic skin homograft sensitivity (accelerated rejection) in humans can be accomplished with DNAse-treated blood leucocyte extracts when obtained from donors sensitized in an adequate fashion. The sensitivity transferred can be shown to be both prompt and enduring. Active hypersensitization of leucocyte donors by repeated sequential graft application may be accompanied by desensitization of the donor to the graft and desensitization of donor leucocytes, resulting in failure to transfer sensitivity.

In adapting the highly sensitive transfer system in human species
to an evaluation of possible mechanisms of homograft rejection, the biological functions of transfer factor have been extended, and the principles elucidated with other states of delayed sensitivity may be, when warranted, transposed. This type of experimental approach gives promise of delineating the antibody or antibodies concerned in orthotopic skin homograft rejection and perhaps a means of investigating the cell type or types involved.

REFERENCES

DISCUSSION

Converse: In a nine-year collaboration with Drs. Lawrence, Rapaport, Thomas, Tillett and Mulholland on this project, we have done 105 grafts on a group of normal human subjects, mostly medical students: of these, 71 were first-set grafts, 18 repeat-set grafts and 16 "white graft" reactions. In reviewing these 105 grafts we began to notice certain differences concerning the individual specificity of the homograft reaction in man that differed from that observed, for example, in the reaction between two strains of inbred mice. There was considerable disparity in the duration of many of the first-set grafts. There was also some disparity in the survival times of control grafts. To investigate this, two experiments were done, which I shall describe briefly.

In the first experiment two recipients (M. and H.) received two successive grafts from N. The first graft was put on and then rejected, and 15 days after the first a second graft was placed. The day after the rejection of this second graft, which underwent accelerated rejection (4-5 days), a third graft was placed, along with six control grafts from different donors. There were five accelerated rejection reactions and two 9- or 10-day survivals. The white graft reaction occurred because of my impatience at the length of time that it took to perform four successive grafts. We attempted to accelerate the interval between the grafts and obtained this white graft reaction, which was interpreted by our group as being an exacerbated state of hypersensitivity which prevents the ingrowth of the vessels.

A further experiment was set up as follows: a graft was placed on nine recipients within the first 5-day interval after the rejection of the first graft, and in all the nine cases there was a typical white graft reaction. In control grafts placed at the same time as the white grafts five of them had an accelerated rejection reaction.

So I am just submitting for your consideration the possibility that in man individual specificity is not as general as it is in the animal. Perhaps this system offers a little glint of hope in the possibility of some degree of classification of tissue groups in the future.
Silvers: In the work that you have just mentioned, there is very good evidence that at least some antigens are shared by some medical students. However, in the case of the work presented by Dr. Lawrence, I don’t think his results ever indicated the existence of shared antigens. What is your explanation for this?

Converse: It must be remembered that the effects of cell transfer on the “test” grafts were judged as significant only in terms of their relation to the behaviour of control grafts on the same recipient. For this reason, it would appear that the bearing of such variables as genetic disparity upon the observations reported by Dr. Lawrence was greatly diminished. The focus in the cell transfer study was on individuals. Evidence of cross-reactions begins to appear when an entire group of subjects is studied, and this was not the end point of Dr. Lawrence’s experiments.

Lawrence: It should be clearly understood that accelerated rejection of unrelated “control” first-set skin grafts requires prior hypersensitization of the graft recipient with the test graft so that it elicits a white graft reaction. This manoeuvre will elevate the intensity of the homograft reaction, not only to the test graft itself, but carry over to the control grafts. Rapaport, Converse, Thomas and I have also found that if leucocytes (or their extracts) are used instead of an orthotopic skin homograft to sensitize, they appear to function as even more potent antigens. The latter mode of sensitization will bring out the phenomenon of sharing of transplantation antigens even more regularly and to a greater degree, that is to include more individuals. There is quite a difference between this sort of deliberate hypersensitization procedure and merely placing one test and one control graft on an individual who has had a cell transfer.

Brent: Dr. Lawrence, if your material is neither an antibody nor an antigen, what is it?

Lawrence: We do not know the biochemical nature of transfer factor. It is, however, a material that transmits immunological information to human recipients.

Brent: I would like to add that with Dr. Lawrence we at University College have tried very hard to get this transfer factor to work in the guinea pig and that this attempt has completely failed. Dr. Lawrence, I believe that you have more recently been able to transfer reactivity in
guinea pigs, using the tuberculin system in place of the homograft system. Is this correct?

Lawrence: The transfer with leucocyte extract in animals was one of the reasons for raising the point about sensitization and desensitization occurring in parallel. In the experiments we did in inbred strains of guinea pig, with you and Prof. Medawar, in attempting to hypersensitize the animal with lymph node cells as homograft antigen, flare-up of all the previous sites of injection of antigen occurred with subsequent desensitization. Our approach in the guinea pig using the tuberculin system viewed the failure to transfer with leucocyte extracts in animals as not necessarily a result of species difference but rather some quantitative aspect of the system which we don’t fully understand as yet. In most reported attempts at transfer with extracts the donor animals have received one course of sensitization. Our notion involved the possibility that repeated hypersensitization with tubercle bacilli might allow the transfer of sensitivity with extracts. When we tried this approach we found the apparently positive initial results few and not consistently reproducible. We also have serious reservations about the meaning of the scattered positive reactions occasionally observed in our guinea pigs, in view of Chase’s finding that leucocyte extracts alone can enhance the reactivity of guinea pig skin to unrelated delayed allergens. We do not believe the use of extracts is an impossibility in the guinea pig, but rather no one has yet hit upon the appropriate means of accomplishing it.

Medawar: We often speak rather glibly about “hyperimmunizing” animals against isoantigens but I don’t know of any circumstances under which one can hypersensitize them: for example, the repeated injection of animals with foreign lymphoid cells doesn’t hypersensitize them, it hyperimmunizes them: they become, during the course of this process, less sensitive. And I don’t really know of a method of making animals hypersensitive to transplantation antigens. If we could do that, I should imagine it might be possible to reproduce your phenomenon in a guinea pig.

Lawrence: In respect of the phenomenon I would say that the capacity to transfer tuberculin-type sensitivity with leucocyte extracts in the guinea pig is still an unsettled question despite much negative evidence having been secured. I would, however, stress that we surely do not
feel on the basis of our unpublished observations that we have succeeded or are about to succeed where others have failed. The guinea pig transfer system does not compare with the ease with which leucocyte extracts transfer sensitivity regularly in the human being. And, secondly, I wonder whether the incorporation of the tissue antigens into the foot pad of the guinea pig in Freund's adjuvant might be more conducive to hypersensitization rather than hyperimmunization.
PROTECTION AGAINST RUNTING BY SPECIFIC TREATMENT OF NEWBORN MICE, FOLLOWED BY INCREASED TOLERANCE*

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When a foetal or newborn mouse of a given inbred strain receives intravenously injected spleen cells, from an adult homologous mouse, the young mouse is modified in such a way that it often becomes unable to reject a tissue graft from the homologous donor strain. This outstanding phenomenon of specific acquired tolerance to living cells is unfortunately often impaired by the appearance, during the second and third week following the treatment, of a typical disease known as “runting syndrome” or “homologous disease” which is thought to be the consequence of an immunological reaction of the immunologically competent injected cells against the vulnerable homologous host. This regrettable phenomenon raises practical and theoretical problems.

From a practical point of view it precludes the application to the human species of the methods used to induce acquired tolerance to living cells, until homologous disease can be completely mastered. This can be done in two ways: one is to protect the newborn by injecting it with antiserum specifically directed against the injected cells (Siskind and Thomas, 1959). Unfortunately this procedure results in the destruction of the injected

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† Director, Raoul Kourilsky.
cells and the absence of induction of tolerance. The second way, which does not present this inconvenience, is to inject the newborn with embryonic, non-immunologically competent homologous cells which will eventually become tolerant to the host (Simonsen, 1957) or to inject a newborn mouse with hybrid parental spleen cells, genetically unable to react against the newborn host. But this is not always easy to do and it cannot be applied to non-inbred animals. As a consequence, the usual way of inducing experimental specific acquired tolerance is still to inject homologous adult spleen cells, knowing that one has to pay tribute to runting disease.

From a theoretical point of view, today the problem is not so much to explain why and how the homologous disease sets in, but rather the reverse problem: since the injected cells are known to persist indefinitely in the homologous tolerant host (Billingham and Brent, 1959); since many of these cells are immunologically competent; and since it is known that they are able to react immunologically against the host, why do they not always do this? Or, if they do, why is the reaction so often well tolerated by the host (Voisin, 1962)?

The present investigation was undertaken in an effort to answer these questions. It is clear, indeed, that classical concepts of homograft rejection reaction and of acquired immunological tolerance do not provide a satisfying answer, unless they are modified in some way. One of the required modifications would be the possibility for some of the injected cells from an adult homologous donor (presumably the “stem cells” which have embryonic potentialities) to become tolerant to the young recipient. A second required modification would be that the injected immunologically competent cells which have already started a homotransplantation reaction against the young host be eliminated in one way or another, the more interesting theoretical suggestion in this respect being the concept of “exhaustive sensitization” (Simonsen, 1960). However, the very existence of the runting
syndrome does not much favour a mechanism which would automatically prevent that syndrome because of the overwhelming quantity of homologous antigens encountered by the injected cells. For this reason and on the basis of theoretical considerations, it was thought of interest to see whether or not another phenomenon known in the field of transplantation would give a clue to the questions under investigation; namely the enhancement phenomenon or phenomenon of immunological facilitation* of the graft by the immunologically competent cells (usually of the host) themselves. This phenomenon is well known (Casey, 1941; Snell, 1954; Kaliss, 1957a): it allows tumours indigenous to one strain of mice to grow successfully when grafted in an adult homologous H-2 histo-incompatible mouse which was previously actively or passively treated for enhancement. Active treatment involves immunization with tissues (usually lyophihized) from the future donor’s strain; passive treatment is realized by an injection of serum from an actively pretreated animal. This phenomenon of immunological facilitation seems to be a general phenomenon since it seems to exist also in rats and rabbits. It applies to many tumours, but not to all (Snell, 1957a). It seems to apply also to normal tissues (Snell, 1957b) in spite of two facts: first, that passive transfer of immunological facilitation has not been firmly demonstrated in cases of normal tissue grafts; and, second, that immunological enhancement results only in some degree of prolongation in the survival time of a normal tissue homograft. This was to be expected because of the much weaker growth potential of normal tissues as compared to tumour tissues. But this concerns normal adult tissues; one may wonder whether embryonic tissues might not react more like tumour tissues do. Actually there are striking analogies between embryonic tissues and tumour tissues (Hamperl, 1956; Bernhard, 1961). These analogies are of

* “Immunological enhancement” and “immunological facilitation” will be used indifferently with the same meaning. “Facilitation” seems a better term because it can be applied to normal tissues as well as to tumour tissues.
two orders: morphological and physiological. From a morphological and ultrastructural point of view they have the following features in common: basophilia due to free ribosome granules without much endoplasmic reticulum; sparse, swollen mitochondria; weak cellular polarity; irregular cellular outlines; high nucleus-cytoplasm ratio; and hypertrophic nucleoli. From a physiological point of view, they are both characterized by: high anabolic activity; rapid rhythm of cell reproduction with numerous mitoses; relatively low degree of differentiation as compared to normal adult tissues; high pinocytotic activity, apparently reflecting similar surface properties; and, finally, great plasticity and adaptation power. From these numerous similarities, the possibility arises that, as far as immunological facilitation is concerned, embryonic tissues may behave somewhat like tumour tissues.

All the preceding considerations led us to try to prevent newborn animals from running by enhancing them in the same way as transplanted tumours are allowed to grow by enhancement. The present work is concerned with passive enhancement of the newborn submitted to injection of homologous adult spleen cells. This, if successful, was thought to have a double interest: theoretical, by showing the possible mechanism by which running is normally prevented; and practical, by providing a way of preventing running and eventually obtaining an increased ratio of tolerant animals as compared to the number of treated animals.

**GENERAL DESIGN OF THE EXPERIMENTS**

The general design of the experiments is rather simple: two strains of mice, A and CBA are schematically involved. Antisera against strain-A lyophilized tissues are prepared in strain-CBA adult mice. Strain-A newborns are injected with strain-CBA adult spleen cells and with CBA anti-A sera in minute, presumably enhancing, doses.
Newborn A’s are followed and examined in the first weeks for runting and over several months for acquired tolerance.

MATERIAL AND METHODS

I. Material

A. Animals

The present experiments involved 101 litters (577 newborn mice). About 300 adult mice were used as donors of spleen cells, as skin donors, as breeders, as producers of antisera, or as donors of red cells. In addition, 50 newborn mice were used to test the toxicity of the sera utilized. Inbred strains of mice were used. The CBA and A/Jax strains were supplied by the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and a stock of CBA mice was kindly supplied by Prof. P. B. Medawar from London in 1958. Both strains were bred in our laboratory.

Isografts performed on the CBA strains of Bar Harbor and London origin demonstrated a 100 per cent tolerance between the two stocks. The mice supplied by Prof. Medawar and their offspring proved to be easier to handle and more resistant. The animals were kept on a standard diet; food was supplied by a French commercial firm (U.A.R.). All the newborn mice used were treated before the 24th hour after birth.

B. Injected materials

These were tissues presumed to contain enhancing antigens, presumed enhancing antisera and cells used to induce acquired tolerance.

(1) Tissues presumed to contain enhancing antigens

Two kinds of tissue were used.

(a) Newborn A mice: killed less than 24 hours after birth. Guts and lungs were discarded and the bodies weighed. Four ml. of physiological saline was added to 1 g. of newborn. Five freezings
and thawings (−70° to +37° c) were rapidly performed in succession and the material ground 1 min. with an Ultraturrax grinder and subsequently submitted to 10 more freezings and thawings. After lyophilization, the vacuum-frozen-dried material was stored (at −20° as a further precaution) in separate ampoules corresponding to 4 ml. of the original suspension. Just before use, the lyophilized powder was diluted with the corresponding volume of distilled water.

(b) Spontaneous tumours indigenous to strain A were also used as immunizing material. The one which was mainly used was a spontaneous abdomino-thoracic tumour, the nature of which unfortunately remained unknown.

(2) Sera presumed to contain enhancing antibodies

These sera were of two types and will be referred to as AN-BI, II or III (anti-A-newborns) and AN-B+T (anti-A-newborns and anti-A-tumours).

(3) Homologous living cells injected into newborn mice

These cells were always CBA spleen cells. Adult CBA mice were splenectomized and the splenic pulp was gently pressed out of the capsule into a Petri dish containing physiological saline. A homogeneous suspension was obtained by projecting the cells repeatedly with a Pasteur pipette on the wall of the dish. The suspension was then spun down in a refrigerated centrifuge (+4°) at a speed of 800 rev./min. for 10 min. The supernatant was discarded and the cells resuspended in an adequate volume of heparinized saline adjusted to a concentration of 120–160 million cells per ml. of suspension. Larger particles settled out by sedimentation within 3 min.

C. Materials to test the sera

The dextran solution was Intradex, kindly supplied by Glaxo Laboratories. Other materials were as described under Methods.
II. Methods

A. Preparation of facilitating antisera

CBA adult mice received tissues from A-strain mice, either normal lyophilized tissues or living tumour tissues.

(1) Normal lyophilized tissues

The immunization schedules of animals receiving lyophilized A newborn and spleen tissues corresponded to that used by Kaliss (personal communication) for lyophilized tumour or splenic tissues. Five injections of 0.5 ml. suspension each were injected intraperitoneally at regular intervals within 3 weeks. Blood was withdrawn 10 days after the last injection and sometimes once more 4 days later. Occasional booster injections (up to 4) were followed by bleedings 5 to 7 days later, with sometimes one more bleeding 4 days later.

(2) Living tumour tissues

Subcutaneous inclusions of approximately 200 mg. of living, non-infected, non-necrotic tumour tissue were performed on CBA recipients and the animals bled 10 and 18 days later. By this time the transplanted tumours had stopped growing and after 20 days a regression was observed. Blood was drawn through a perpendicular incision of the caudal artery. The sera obtained were stored at −20°.

B. Tests for activity of the antisera

(1) In vitro: haemagglutination

Gorer's haemagglutination test (Gorer and Mikulska, 1954) was adopted, using as medium absorbed human serum with dextran. The human serum was absorbed at 4° first with rat red blood cells (RBC), as suggested by Amos (personal communication through Dr. Haward), then with miscellaneous (various H-2 alleles) mouse erythrocytes. We found it technically more convenient to mix the A-strain RBC-CBA serum dilution
directly on a Kline agglutination plate, using precautions to avoid drying (Gorer mixed the serum dilutions with RBC in tubes and placed a drop from each tube on a separate slide for microscopic observation). The examination was made under a binocular magnifying glass and the results graded as $-$, $\pm$, and $+$ to $++$+. The highest dilution giving $+$ is taken as the strength of the serum, and the dilutions giving the strongest agglutination are recorded in parentheses. This leads to notations such as 512 ($+++64-128$).

(2) *In vivo*

Sera were tested both for cytotoxicity and enhancing power. Dilutions of sera corresponding to volumes of pure sera ranging from 0.20 to 2.00 mm.$^3$ were injected intravenously in newborn A mice.

(a) *Cytotoxicity* was studied by weight curve and mortality rate of A newborns injected with sera alone.

(b) *Enhancing power* was evaluated by the ability of sera to protect A newborns against runting by homologous (CBA) adult spleen cells. Protection was judged by weight curve and mortality rate of the treated animals as compared to animals having received the same amounts of CBA cells and normal CBA serum.

C. Injections of the newborns with homologous cells

Doses of 7±1 million CBA adult spleen cells were injected in a volume of 0.05 ml. into the orbital branch of the anterior facial vein of newborn A mice according to Billingham, Brent and Medawar's technique (Billingham and Brent, 1956). Paraffin oil was applied over the retro-orbital region in order to increase the transparency of the skin as well as to magnify the vein. A total of 471 newborn A mice were injected intravenously during the present experiments. Occasional post-injection breathing troubles were overcome in most cases by repeated gentle pressure
on the abdomen and thorax of the newborn. During the first week after birth, parental cannibalism caused the complete loss of 18 litters, amounting to 81 newborns among which 69 had been injected.

D. Facilitation of the newborns

Appropriate dilutions of the presumed enhancing CBA sera were injected intravenously into the A newborns. Identical dilutions of normal CBA sera were injected in a similar way in other A newborns of the same litters. The doses of serum injected were evaluated in cubic millimetres of pure serum; they usually varied from 0.20 mm.³ to 1.00 mm.³. Additional newborns of the same litters received nothing and served as normal controls. For reasons of technical convenience, the CBA sera (either enhancing or normal) were mixed with the CBA spleen cells before being injected into A newborns. In some experiments one to three additional injections of CBA sera (either enhancing or normal) were performed on the animals within the first two weeks of the animal’s life using the intraperitoneal route.

In summary, each litter of A newborns was divided into three parts: the first group received CBA spleen cells plus CBA enhancing serum; the second group received the same amount of the same preparation of CBA spleen cells plus CBA normal serum; the third group received either nothing or physiological saline at the same volume, and served as normal controls.

E. Occurrence of runting

Occurrence of runting was evaluated by regular weighing of all the animals in all the litters on days 7, 10, 14, 21, 28 and 35 after birth. Weight curves were established for each particular experimental situation. The intensity of the homologous syndrome was also noted according to the general condition (diarrhoea, alopecia, asthenia, etc.).
Cumulative mortality curves were also established. No systematic pathological study was attempted.

**F. Test for specific acquired tolerance**

Occurrence of specific acquired tolerance was tested by skin grafts performed at the age of 6–8 weeks on the A mice injected at birth with CBA spleen cells (plus enhancing or normal CBA serum). The grafts were full-thickness CBA female skin; they were circular, shaped with scissors, with a diameter of approximately 16 mm. and were fixed on a previously prepared bed over the stratum carnosum with interrupted stitches using “Sertix” oo silk suture thread. A piece of saline-moistened hydrophilic cotton was applied and an adequate pressure assured by means of an elastic “Sealtex Latex Bandage”.

Six days later the bandage was removed and by this time the grafts were usually well established and vascularized, the edges had joined together and the stitches were easily removed with the cotton to which they adhered. Grafted animals were placed in separate cages and observed for several months, often throughout the normal life span of the animal. No attempt was made to determine the histological picture of the epidermis at any time since the expected increase in survival time was more than the normal survival time (10 days) observed in unprepared animals of the same strain combination. Such an increase in survival time (21 days) was required before one could speak of tolerance. Partial tolerance was considered as concerning survival time of from 21 days to 100 days. When a graft started to undergo changes before 100 days and was rejected after 100 days, it was considered as a case of partial tolerance. The only animals considered as permanently tolerant were those which, after 100 days, bore healthy grafts with normal hair growth of the same agouti color as the original donors and growing in a direction which differed by 180° from that of the surrounding host fur. It has been our constant experience that grafts which were in that healthy
state after 100 days were never spontaneously rejected. For that reason, several tolerant animals were killed after four months for other experiments. However, many tolerant animals were kept under observation for periods up to 13 months.

G. Controls
Various controls were set up at different steps of the experiments.

(1) Histocompatibility
Histocompatibility was repeatedly tested by skin grafts. Intrastain grafts were uniformly accepted even between the CBA mice from Bar Harbor and from London while inter-strain grafts between A and CBA were rejected in all cases within $10.5 \pm 1$ days in both strain directions.

(2) Viability of injected spleen cells
Viability was ascertained by high incidence of runting and high percentage of tolerance. Viability tests with eosin were used at the beginning and then discontinued.

(3) Activity of antisera
Whatever test was used (haemagglutination, toxicity or facilitation) the immune activity of CBA sera was controlled by similar dilution of normal CBA sera which remained invariably devoid of activity.

(4) Additional controls in the study of protection against runting
(a) CBA immune sera alone were tested at various dilutions. Dilutions non-toxic for the newborns were used.

(b) As some variations were noticed in the weight curves of untreated litters over different periods of the year, we kept uninjected controls under observation and their evolution was compared with that of experimental animals. Only the litters in which no death at all was observed among these uninjected controls from day 7 to day 35 were accepted for study. This led
us to discard 8 litters, amounting to 46 newborns among which 35 were injected.

(c) Some of the normal controls (25 per cent) received intravenous injections of physiological saline at birth, in the same volume as experimental animals received sera + cells.

(d) From repeated preliminary experiments it was known that A newborns injected with isologous adult spleen cells behave (as far as their state of health, weight curve and survival were concerned) the same way as uninjected newborns or newborns injected with saline. It was therefore not deemed necessary to add that type of control to litters already divided into three series.

RESULTS

Each of 101 litters comprising 577 newborns of strain A was divided into 3 series. The first series (250 newborn mice) received adult CBA spleen cells plus immune serum prepared by immunizing adult CBA against A-strain tissues. The second series (221 newborn mice) received the same number of CBA spleen cells and the same amount of normal CBA serum. The third series (106 newborn animals) received nothing or physiological saline and was followed as normal controls. It must be remembered that 18 litters (81 newborn animals) were lost before the 7th day owing to the cannibalism of the parents; 8 more litters (46 newborns) were discarded because one or more of the normal controls included in these litters died during the experiments. Finally 75 litters comprising 450 newborn animals were considered as satisfactory and were utilized for this study. They were followed and studied for occurrence of runt disease and, later, for specific tolerance to CBA skin. The immune sera utilized to treat these animals were also studied.

The experimental results will be reported in three sections: the study of runting and its prevention; the immunologically specific properties of the immune sera; and the study of tolerance and its enhancement.
I. Protection against runting

The occurrence of runting was followed by weight curves and mortality rate as well as by the well known symptoms characteristic of this condition. The percentage of typical runting was found to be particularly high in our experimental conditions as shown by the percentage of deaths on day 35 (67 per cent) in the animals of series number 2 (homologous cells plus normal serum). For reasons of convenience, the data concerning the mortality rates will be presented first, followed by the data concerning weight curves.

A. Mortality rates

(i) Overall results

The results obtained with the various sera, utilized at various doses, once or several times, will be reported first in overall figures comprising all the data. The absolute numbers of deaths as well as the percentages of deaths are determined from the number of animals alive on day 7 after birth. This is the usual way to proceed, owing to the fact that mortality before day 7 may be due to several factors other than runting. However, it was found of interest to consider also the figures on day 0 (day of birth and of injection). To do this, the figures concerning day 0 were plotted against the figures concerning day 7; the day 0 figures were plotted as negative entities. This was done in the tables as well as in the graphs. The overall figures are summarized in Table I and Fig. 1. From these, it can be seen that there is a striking and highly significant protection against runting in series 1 (animals treated with "facilitating" antiserum). It can also be seen that this protection consists both in a delayed appearance of runting and in a decrease in the percentage of animals runted. Another point concerns what happens between day 0 and day 7; it will be considered later. Consideration of Fig. 1 leads to the observation that protection against runting is maximum between
Table I.

OVERALL FIGURES CONCERNING THE PREVENTION OF RUNTING BY SPECIFIC TREATMENT (FACILITATION) OF NEWBORN MICE

Number and percentage of deaths as compared to Day 7 after birth:

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>-21</td>
<td>149 an.</td>
<td>19</td>
<td>50</td>
<td>84</td>
<td>92</td>
<td>99</td>
</tr>
<tr>
<td>(-14%)</td>
<td>0 (0%)</td>
<td>(13%)</td>
<td>(34%)</td>
<td>(56%)</td>
<td>(62%)</td>
<td>(67%)</td>
</tr>
<tr>
<td>-13</td>
<td>184 an.</td>
<td>6</td>
<td>15</td>
<td>50</td>
<td>72</td>
<td>76</td>
</tr>
<tr>
<td>(-7%)</td>
<td>0 (0%)</td>
<td>(3%)</td>
<td>(8%)</td>
<td>(27%)</td>
<td>(39%)</td>
<td>(41%)</td>
</tr>
</tbody>
</table>

Animals treated at birth with homologous cells and:
Normal CBA serum

Immune (facilitating) CBA serum

Significance of the observed differences in percentages: $t = 2.10$
Probability that the observed differences are due to chance: $3.6 \times 10^{-2}$

Cumulative numbers and cumulative percentages of death calculated from day 7. For negative figures on day 0 see text. All differences are statistically significant.
day 7 and day 14, at the time when runting is maximum in unprotected animals. Between day 14 and day 21 it is less powerful, while the rate of death by runting is decreasing in unprotected animals. Between day 21 and day 28 the protection of treated animals is much less powerful due to the fact that, while their death rate is about the same as the week before (or a little less), the death rate of unprotected animals has now strikingly decreased.

Finally, between day 28 and day 35 there is almost no death in protected animals, while the death rate of unprotected animals is about the same as the week before. All the preceding information

![Diagram of cumulative curves of mortality](image-url)

**Fig. 1.** Cumulative curves of mortality (expressed in percentage of deaths) starting at day 7. The number of animals alive on day 0 has been compared to the number of animals alive on day 7 and expressed as negative entities (see text). Number of animals alive on day 7: 184 “protected” (i.e. injected with homologous cells plus facilitating serum); 149 “unprotected” (i.e. injected with homologous cells plus normal serum); 76 normal controls. For statistical significance of the differences see Table I.
Fig. 2. Showing complete littermates of A-strain series on day 35 (without mortality in this particular case). Mouse T (normal control) weighs 13.3 g. Mouse C+S(f) (treated at birth with CBA spleen cells + facilitating CBA serum) weighs 10.4 g. Mice C (treated at birth with CBA spleen cells + normal CBA serum) weigh respectively 9.5 and 6.2 g.
may be considered with confidence because of the high significance of the figures and the large number of animals studied (see Table I). Fig. 2 gives a good idea of what a typical litter looks like on day 35.

To analyse these results further, it is necessary to study the factors of variation affecting the observed protective effects against runting.

(2) Factors of variation

These factors of variation appear to be concerned mainly with the type of serum utilized for protection, with the dose of serum injected and with the number of injections.

(a) Type of serum. Four pools of sera were utilized in a significant

![Diagram of cumulative curves of mortality](image)

**Fig. 3.** Cumulative curves of mortality (same method as in Fig. 1). Protected animals have been treated with serum AN-BI, a serum of low activity; protection is limited to a delayed occurrence of runting. Number of animals alive on day 7: 42 protected; 39 unprotected; 23 normal controls.
number of litters and have been compared: AN-BI, AN-BII, AN-BIII and AN-B + T. Lyophilized tissues of newborn A mice were used for immunizing injections in adult CBA mice; the individual doses of each immunizing injection were: $0.25$ ml. in animals furnishing serum AN-BI; $0.30$ ml. for AN-BII; $0.50$ ml. for AN-BIII and AN-B + T. The animals furnishing this last serum received a further graft of tumour A.

The protective activity of these four sera was as follows: AN-BI was the least powerful, by far. As can be seen in Fig. 3, its action was only to delay the appearance of runting, because the number of animals finally dying in series one (facilitating serum) and series two (normal serum) was almost the same, as judged on day 35. Serum AN-BII gave intermediate results which were very much like the overall figures presented above (Fig. 1). As for sera AN-BIII and AN-B + T, they were the most powerful; they are considered together (Fig. 4) in order to have a number of animals similar to that for serum AN-BI. The protection afforded by sera AN-BIII and AN-B + T is impressive and seems to begin very early. Nevertheless, even in this case, there is some remaining degree of runting which leads to the death of several animals between day 21 and day 28.

It should be noted that sera withdrawn from the same mice at different periods of immunization or even at intervals of a few days do not always seem to have the same degree of activity.

Another important fact concerning the quality of the sera is that several freezings and thawings of the sera result in a decrease of their protective power and require an increase in the doses used.

(b) Dose of serum. The influence of the dose of serum on the degree of protection against runting has not been systematically studied. Nevertheless, we have relevant data concerning serum AN-BI, which was used at four doses: $0.20; 0.25; 0.50$ and $1.00$ mm. While doses of $0.20$ and $0.25$ had no protective effect, but only caused a slight retardation in the occurrence of runting, doses of $0.50$ and $1.00$ had both a retarding effect and definite pro-
tective effect. It seems, therefore, that below a threshold dose, enhancing sera have no striking activity.

(c) Number of injections. The influence of this factor was studied with serum AN-BII: 57 animals received only 1 injection (0.20 or 0.25 mm.³) at birth; 52 animals received a similar

ANIMALS TREATED WITH:

- Homologous cells + normal serum
- Homologous cells + serum
- AN-B III or AN-B+T
- Nothing (normal controls)

Fig. 4. Cumulative curves of mortality (same method as in Fig. 1). Protected animals have been treated with sera AN-BIII or AN-B+T, two sera of high activity; protection is both relative (delayed occurrence of runting) and absolute (great decrease in the final proportion of deaths). Number of animals alive on day 7: 40 protected; 33 unprotected; 18 normal controls.

injection at birth, followed, between day 5 and day 17, by one to three further injections (0.20 to 1.50 mm.³), the dose being increased with the age of the animals. Eighty-eight animals received normal serum at birth and some of them received additional injections of normal serum. Finally, 40 animals received
nothing and were followed as normal controls. The results are shown in Fig. 5, where it can be seen that the repetition of injections had a moderate but definite additional protective action over the single injection. This additional protective action seems to be effective mainly between day 14 and day 28. This is precisely

![Cumulative curves of mortality](image)

**Fig. 5.** Cumulative curves of mortality (same method as in Fig. 1). Increase in protection by one to three additional injections. Number of animals alive on day 7: 54 protected (one single injection); 52 protected (one to three additional injections); 81 unprotected; 40 normal controls.

the period during which runting is maximal in animals treated with one single injection of facilitating serum at birth.

(3) **Chronology of runting**

The main facts concerning the chronology of runting in both series treated with homologous spleen cells plus either normal CBA serum or facilitating CBA serum, can be best expressed and analysed by means of histograms giving the number of deaths per
day of age. In order to make comparison between the two series easier, the number of deaths has been expressed in terms of percentage in Fig. 6. The figures of these histograms and some other experimental facts provide information which should be useful to the understanding of the mechanisms both of runting and the protective activity of facilitating sera. The information concerns the events happening respectively before and after day 7.

(a) Before day 7. It is generally considered that deaths taking place before day 7 cannot be ascribed to runting because there are other factors which may increase the mortality during this period. This is true indeed: 18 of our litters (81 newborns) had all been eaten before day 7, whether the newborns had been treated or not. However, among the 75 litters kept for study there had been a different mortality rate according to the series considered: between day 0 and day 7, 7 out of 83 (8·4 per cent) untreated normal animals died and 13 out of 197 (6·6 per cent) animals treated with homologous cells plus facilitating serum died also. They can be considered (as far as mortality rate is concerned) as members of the same population of 280 animals of which 20 (7·1 per cent) died between day 0 and day 7. During the same period 21 out of 170 (12·3 per cent) of the animals treated with homologous cells plus normal serum died. It can easily be calculated that, in this last series, roughly 9 animals (that is 43 per cent of the deaths and 5·3 per cent of the animals) died for a reason which was peculiar to that series—a reason which is interpreted as early runting. This is in agreement with Russell’s (1960) description of runting beginning around day 5. Further significant information given by studying this early period is the total absence of runting in animals treated with enhancing serum.

(b) After day 7. The upper histogram of Fig. 6 shows that, in unprotected animals, the maximum mortality from runting occurs around days 11–12 and that it decreases progressively but has not completely stopped by day 35. The lower histogram of
the same figure shows that, in addition to mortality from runting being much less important in the protected animals, it is also delayed, reaching its maximum around day 18 and decreasing progressively to become almost nil by day 35. It should be noted also that between day 21 and day 28, the mortality rate is signi-

![Histogram showing ratio of percentage of deaths per day of age in two series: unprotected (upper histogram) and protected (lower histogram), between birth and day 35.](image)

ficantly higher in the protected series than in the unprotected series.

However, these differences of chronology between the two series do not affect the clinico-pathological picture of runting, which was exactly the same in the two series in the animals in which it occurred. The picture conformed in every respect to the classical one (Billingham and Brent, 1959).
B. Weight curves

A study of the mean weights has been made for all of the three series (untreated controls, protected animals and unprotected animals) without attempting, so far, to analyse the factors of variation. The overall results are given in Fig. 7. These results show that the weight curve of normal controls is almost a straight line, that the animals double their 7-day weight on
day 15; they triple it on day 26 and they quadruple it on day 34. As for the two series of animals injected at birth with homologous cells, they have weight curves very close to each other and ascending more slowly than that of the normal controls: the mean weights of day 7 have increased twofold on day 20 and threefold on day 30; they have not increased fourfold by day 35. The weight curve of the protected animals is slightly but consistently above the weight curve of the unprotected animals. A statistical analysis of the coefficient of variation of the mean weights in the 21 experimental situations (21 experimental points on Fig. 7) leads to the conclusion that the differences between the three series on a given day are not significant (Table II). This can be easily understood from the great variability in weight of individual litters. This great variability depends on two main factors: the number of newborn animals in one litter and the period of the year (the experiments presented here were carried out over two years). However, if one considers each individual litter one can make the following relevant observations: on days 10, 14, 21, 28, and 35, in 88 per cent (± 3 per cent) of the litters, the untreated normal control animals had a mean weight superior to the mean weight of the experimental animals, while the reverse situation occurred in only 6 per cent (± 1.5 per cent) of the litters (the remaining 6 per cent pertains to the litters where the mean weights were about the same). These figures are, indeed, highly significant. On the other hand, on day 7, in 53 per cent of the litters the control animals weighed more than the experimental ones, while the reverse situation occurred in 7 per cent of the litters: this is highly suggestive of a true difference.

Concerning the much smaller difference between the mean weights of the two experimental series (the protected and the unprotected), it has been found that, on each individual day (7, 10, 14, 21, 28 and 35), the protected animals had a mean weight superior to the mean weight of the unprotected animals in 72 per
Table II

OVERALL FIGURES CONCERNING THE PREVENTION OF RUNTING BY SPECIFIC TREATMENT (FACILITATION) OF NEWBORN MICE

Mean weight in grams of the animals on day

<table>
<thead>
<tr>
<th></th>
<th>7</th>
<th>10</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.29</td>
<td>4.11</td>
<td>5.48</td>
<td>6.85</td>
<td>8.80</td>
<td>11.75</td>
<td></td>
</tr>
<tr>
<td>±0.73</td>
<td>±1.23</td>
<td>±1.67</td>
<td>±1.81</td>
<td>±2.72</td>
<td>±3.30</td>
<td></td>
</tr>
<tr>
<td>V=22%</td>
<td>V=30%</td>
<td>V=30%</td>
<td>V=26%</td>
<td>V=31%</td>
<td>V=28%</td>
<td></td>
</tr>
<tr>
<td>3.39</td>
<td>4.43</td>
<td>5.67</td>
<td>6.88</td>
<td>9.39</td>
<td>12.32</td>
<td></td>
</tr>
<tr>
<td>±0.76</td>
<td>±1.21</td>
<td>±1.62</td>
<td>±1.93</td>
<td>±2.79</td>
<td>±3.41</td>
<td></td>
</tr>
<tr>
<td>V=23%</td>
<td>V=27%</td>
<td>V=29%</td>
<td>V=28%</td>
<td>V=30%</td>
<td>V=28%</td>
<td></td>
</tr>
<tr>
<td>3.59</td>
<td>5.15</td>
<td>6.82</td>
<td>8.88</td>
<td>12.25</td>
<td>14.97</td>
<td></td>
</tr>
<tr>
<td>±0.85</td>
<td>±1.16</td>
<td>±1.64</td>
<td>±1.75</td>
<td>±2.26</td>
<td>±2.68</td>
<td></td>
</tr>
<tr>
<td>V=24%</td>
<td>V=23%</td>
<td>V=24%</td>
<td>V=20%</td>
<td>V=18%</td>
<td>V=18%</td>
<td></td>
</tr>
</tbody>
</table>

Animals treated at birth with homologous cells and:
Normal CBA serum

Mean weights from Day 7 to Day 35. The sign ± is followed by the value of the standard deviation; V is the coefficient of variation = 1000/m. Apparent absence of statistical significance of the differences observed between the three series. Actual significance (see text).
cent (± 5 per cent) of the litters, while the reverse situation occurred in 21 per cent (± 4 per cent) of the litters (for a typical litter, see Fig. 2). It can be concluded that the observed differences, although small, are probably significant. Therefore, it can be said with some confidence that there is a significant decrease in the mean weights of experimental animals as compared to normal controls, especially after day 7. Furthermore, there is a slight but probably significant decrease in the mean weights of unprotected animals as compared to protected animals. This difference is already noticed (although extremely small: 0.10 g.) on day 7. This effect on weight occurs in addition to the difference in death rate since, obviously, only the living animals are weighed. The two facts that there is a difference in weight and that this difference is a very small one seem to be of importance.

That such a protection against runting may be obtained by doses of "facilitating" serum as small as 0.20 mm. can seem somewhat disturbing. At any rate, it raises the question of the specificity of this action. The following section answers this question.

II. Immunological specificity of the sera

Immunological activity and specificity of the sera were tested both in vitro by haemagglutination and in vivo for immunotoxicity toward A newborn animals.

A. Haemagglutination

Haemagglutination of A red cells—performed according to Gorer's technique, slightly modified (see Methods)—by anti-A sera gave the following results: serum from CBA mice injected only with lyophilized strain-A newborns (sera AN-BI, AN-BII and AN-BIII) gave negative results, while serum from CBA mice injected with lyophilized strain-A newborns and grafted later with A tumour gave positive results, graded as 64 (+ + + 16) or 65 (+ + + 16–32) (see Methods for an explanation of these
notations). However, mice which had such positive sera, and had negative sera after 3 months without further immunization, again had strongly positive sera such as 512 (+ + + 8-32) or 512 (+ + + 16-128) after reinjection with A lyophilized newborns. This fact is of importance, for it demonstrates that lyophilized newborns contain antigens able to induce formation of anti-strain antibodies and, as a consequence, that sera AN-BI, AN-BII and AN-BIII must have contained strain-specific (anti-\(H-2^a\) antigens, particularly D) antibodies, even when they were not able to give direct haemagglutination. On the other hand, this might also explain why sera AN-BI and AN-BII were much weaker than serum AN-B+T.

**B. Immunotoxicity**

The direct action on A newborns of minute amounts (mm.\(^3\) doses) of CBA anti-A sera was ascertained by intravenous injection of these sera alone (without cells) in A newborns, at doses higher than the ones used for enhancement. Serum AN-BIII was particularly studied in this respect. The results of a typical experiment are given in Table III.

From this table, it is clear that serum anti-newborn-A is highly immunotoxic for newborn A. In this particular case the dose closest to the LD\(_{50}\) is 1.00 mm.\(^3\). The amount of serum required for LD\(_{50}\) seems to be in a very narrow range since a dose of 0.80 mm.\(^3\) causes no death and dose of 1.20 mm.\(^3\) causes death of all injected animals.

Doses less than 1.00 mm.\(^3\), although they are not lethal, still have a definite depressive action on the weight curve—a depressive action which seems to be completely overcome by day 15. This immunotoxicity is specific for A-newborn strain-specific antigens. The only control used, so far, has been CBA newborns injected with doses of 2 to 10 mm.\(^3\) of CBA anti-A serum. No mortality occurred from these injections and no decrease in the weight curve. Serum AN-BI, whose protective effect was slight,
<table>
<thead>
<tr>
<th>Dose of ANB-III serum injected in newborns</th>
<th>0.20 mm.³</th>
<th>1.00 mm.³</th>
<th>1.20 mm.³</th>
<th>0.00 mm.³</th>
</tr>
</thead>
<tbody>
<tr>
<td>or 0.40 mm.³ or 0.80 mm.³</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Normal controls)</td>
</tr>
</tbody>
</table>

| Number and proportion of deaths           | 0/9 (0%)  | 4/11 (36%) | 6/6 (100%) | 0/8 (0%)   |

<table>
<thead>
<tr>
<th>Mean weight of the survivors (the chosen unit of weight being that of the corresponding non-treated littermates checked at the same time)</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.91</td>
<td>0.90</td>
<td>0.93</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.77</td>
<td>0.78</td>
<td>0.87</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
had to be used in high doses in order to have a lethal action on A newborns.

The main interest of these results on immunotoxicity obtained with minute amounts of specific antisera is to provide a solid basis for the interpretation of the protective action of the same sera (utilized at lower doses) in terms of immunological facilitation—or specific enhancement.

The last part of this work is concerned with results obtained in the study of specific acquired tolerance of skin grafts.

III. Increase in tolerance

After having been followed for runting, all the survivors of the two series of strain-A animals treated at birth with homologous (CBA) cells (plus either normal or facilitating serum) were grafted with corresponding homologous (CBA) skin and the evolution of the grafts was observed. Following the criteria described under “Methods”, the grafted animals were separated in 5 groups according to the evolution of the grafts. The groups were defined as follows:

Group 0: animals which died between day 0 and day 16 after grafting (10 animals): these animals were discarded as of no use in evaluating tolerance or rejection (no graft was rejected before death in this group).

Group 1: animals which died between day 30 and day 100 (no animal died between day 16 and day 30). All of them (10 animals) had perfectly healthy grafts when they died: they were classified as “undetermined tolerance”, which means that it was impossible to know whether they would have been cases of partial or permanent tolerance, had they survived.

Group 2: animals which rejected their grafts before day 16 were classified as cases of “normal rejection”.

Group 3: animals which rejected their grafts between day 30 (no animal rejected its graft between day 16 and day 30) and day 100 were classified as cases of “partial tolerance”.
Group 4: animals which retained perfect grafts for at least 100 days and never rejected them during their lifetime were classified as permanently tolerant.

Table IV

<table>
<thead>
<tr>
<th>Animals treated at birth with homologous cells and:</th>
<th>No. of animals grafted, surviving over 16 days</th>
<th>Normal rejection</th>
<th>Undetermined tolerance</th>
<th>Partial tolerance</th>
<th>Permanent tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal CBA serum (taken as 100%)</td>
<td>37 (10.8%)</td>
<td>4 (8.1%)</td>
<td>3 (24.3%)</td>
<td>9 (56.8%)</td>
<td></td>
</tr>
<tr>
<td>Immune (facilitating) CBA serum (taken as 100%)</td>
<td>79 (0%)</td>
<td>0 (8.9%)</td>
<td>3 (3.8%)</td>
<td>69 (87.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Significance of the observed differences in percentages: $t =$

<table>
<thead>
<tr>
<th>Probability that the observed differences are due to chance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1%</td>
</tr>
<tr>
<td>$&lt; 0.01$</td>
</tr>
<tr>
<td>$10^{-4}$</td>
</tr>
<tr>
<td>$&lt; 10^{-4}$</td>
</tr>
</tbody>
</table>

All differences—or absence of difference—statistically significant (significance of the difference in rate of normal rejection evaluated by the method of binomial expansion).

It was found that only the animals which received homologous cells plus normal serum were capable (in 4 cases out of 37, 10.8 per cent) of giving a normal rejection, while animals which received facilitating serum instead of normal serum were in no instance capable of a normal rejection. It was also found that the animals treated with facilitating serum were able to develop permanent tolerance in 69 cases out of 79 (87.3 per cent) while the animals treated with normal serum developed it in 21 cases out of 37 (56.8 per cent). The percentage of undetermined tolerance is
Fig. 9. Behaviour of three CBA skin grafts on three strain-A littermates. Appearance on day 33 after grafting. Mouse C (treated at birth with CBA spleen cells + normal CBA serum) shows partial tolerance (graft will be completely rejected on day 49); notice absence of hair on most of the surface of the graft with desquamation. Mouse T (untreated control) shows pigmented remnants of graft rejected on day 10. Mouse C+S(f) (treated at birth with CBA spleen cells + facilitating CBA serum) shows complete viability of the graft (observed up to the eighth month, at which time it was killed).
almost exactly the same in the two series. As for the percentage of partial tolerance, it follows, for obvious reasons, the reverse direction of permanent tolerance. The precise figures and their statistical significances are recorded in Table IV and illustrated in Figs. 8 and 9. It seems impossible, therefore, to escape the conclusion that animals treated with facilitating serum yield a better tolerance (both from the point of view of the number of tolerant animals and from the standpoint of quality of the tolerance) than animals treated with normal serum. Another point worth mentioning concerns the number of animals which died in the two series (facilitated and non-facilitated) between the time of grafting and 100 days after grafting: the number is 16 animals

Fig. 8. Bar diagrams expressed in percentage of the grafted animals in each one of the three series (protected, unprotected and normal controls). The figures at the bottom of each bar represent the actual numbers of animals.
out of 88 (18 per cent) in the facilitated series while it was 3 out of 38 (8 per cent) in the non-facilitated series (difference statistically significant). The meaning of these facts will have to be discussed. Finally, it was thought interesting to consider the practical overall results obtained in induction of tolerance during the course of the preceding experiments.

IV. Practical overall results

Since it is interesting to know the best way to obtain tolerance (and particularly permanent tolerance) to tissue grafts in a given combination of strains, we have compared the percentage and quality of tolerance induced in A newborn mice injected at birth with CBA normal adult spleen cells and injected at the same time with minute amounts of CBA serum either normal or immune against A antigens. The results are expressed in Table V.

Table V

<table>
<thead>
<tr>
<th></th>
<th>Number of animals treated at birth</th>
<th>Number of tolerant animals</th>
<th>Number of permanently tolerant animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals treated at birth with homologous cells and:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal CBA serum</td>
<td>170 (taken as 100%)</td>
<td>33 (19%)</td>
<td>21 (12%)</td>
</tr>
<tr>
<td>Immune (facilitating) CBA serum</td>
<td>197 (taken as 100%)</td>
<td>79 (40%)</td>
<td>69 (35%)</td>
</tr>
<tr>
<td>Significance of observed differences in percentages: ( t = )</td>
<td>4.28</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Probability that the observed differences are due to chance</td>
<td>(&lt; 10^{-4})</td>
<td>(&lt; 10^{-4})</td>
<td></td>
</tr>
</tbody>
</table>

Numbers and percentages of mice tolerant and permanently tolerant as compared to the numbers of mice treated at birth (all survivors were grafted).
This table shows the importance and the extremely high significance of the final yield of tolerance: twice as many tolerant animals and three times as many permanently tolerant animals are obtained in the series of animals which received facilitating serum as compared to animals which received normal serum. The probability that this result might have been due to chance is less than $10^{-4}$.

**DISCUSSION**

The experimental results reported above deal mainly with runting and protection against runting; with immunological facilitation (enhancement phenomenon) and its application to non-tumour tissues; with specific acquired tolerance and increased rate to tolerance. The discussion will also deal successively with these three questions.

I. Runting and protection against runting

A. What are the normal course and the mechanism of runting?

In the experiments reported, runting occurred (in unprotected animals) rather early—before day 7—and reached its maximum (as judged by death rate) around day 11 to 12, to decrease progressively, rapidly at the beginning (between day 14 and day 21) and more slowly afterwards. It had not completely disappeared (or, at least, it lethal consequences) on day 35. These facts may have implications for the mechanism of runting: they are quite compatible with the generally accepted view that the basic mechanism of runting is of the same nature as the mechanism of homograft rejection. However, the following differences have to be noted: the time of maximum severity of runting as well as the end of its active phase are somewhat delayed as compared to homograft rejection; furthermore its lethal consequences can be observed from around day 5 to later than day 35. These
alterations are compatible with the view that at least as early as day 5, and continuing thereafter, runting is counteracted by some mechanism which will have to be explained.

B. What is the basic mechanism by which immune sera exert protection against runting?

Immune anti-newborn sera do possess a definite (sometimes strikingly strong) protective activity toward newborns of the same strain injected at birth with homologous adult spleen cells. This activity cannot be ascribed to mechanisms such as antibacterial activity for instance, for obvious quantitative reasons; furthermore, if it was the case, it would mean that runting is a bacterial disease, which does not seem reasonable. On the other hand, this protective activity is confined to sera having special immunological properties: utilized at “high doses” they are immunotoxic for the corresponding homologous newborns, while they are innocuous to isologous newborns. Furthermore these sera possess a specific haemagglutinating power towards red cells of the same strain as the newborns. When they do not possess this activity, there are reasons (see results on haemagglutination) to think that they possess an immunological activity of a comparable order. These different properties of the sera utilized in these experiments—namely protective or preventive activity against a reaction analogous to homotransplantation reaction, immunotoxicity at higher doses and haemagglutinating power—are precisely the properties characteristic of sera able to produce passive immunological enhancement (or facilitation) of transplanted homologous tumours (Kaliss, 1957a; Gorer, 1958). For all the preceding reasons it is reasonable to assume that the basic mechanism by which these sera protect the corresponding newborns against runting is the same as the one by which enhancing sera protect the corresponding tumours against rejection reaction. This was indeed the working hypothesis which led us to perform the reported experiments (Voisin, 1958, 1960).
C. How can passive facilitation counteract runting?

This question may remain without an answer for some time. There are only two things that we can say at present: first, that one cannot see any reason to consider the operative mechanism as different from the one at work in conventional immunological enhancement (Kaliss, 1957b); and, secondly, that the "afferent hypothesis" (Billingham, Brent and Medawar, 1956) does not seem able, alone, to take into account the fact that reinjections of facilitating serum result in an additional protection of newborns—a fact which is consistent with the observation that enhancing sera can protect homologous transplanted tumours, even when these sera are injected several days after transplantation (Kaliss, 1957a). After years of study, the question is still open to discussion as to whether enhancing antibodies have a peripheral action, coating the antigens and preventing them from reaching, unmodified, the immunological centres and/or protecting them against an already established immunological reaction, or a central inhibitory action on the immunological centres themselves: it seems indeed possible that a specific antibody might be able to prevent the immunological centres from responding to an antigenic stimulus (toward the corresponding antigen), especially in the direction of delayed hypersensitivity. In any case, a precise knowledge of the mechanism of facilitation would be of great theoretical and practical value.

D. Is active facilitation the normal process leading to protection against runting?

It was mentioned at the beginning of this report that one of the most puzzling questions was: why do immunologically competent homologous cells injected into newborn mice not always react immunologically against the newborns or, if they do, why is the reaction so often well tolerated by the host? There are only two theoretical possibilities: either the newborn animal reacts
actively against the homologous immunologically competent injected cells or it undergoes passively the consequences of the immunological reactions of the injected cells—but these reactions are peculiar as they do not always lead to runting. Since runting begins very early and already has lethal consequences at a time when the newborn is still incapable of immunological defence one is led to think that—at least for a period of several days after birth—the second possibility is the operative one. What is then the mechanism by which injected cells are prevented from runting the host? One may think that part of the injected cells (presumably stem cells) become tolerant to the host. But this would not prevent the other cells from reacting against the host. One may then invoke a hypothetical process of "exhaustive sensitization" (Simonsen, 1960) according to which, when injected cells are sensitized enough to the antigens of the newborn, they are killed by contact with surrounding host antigens, the only surviving cells being the ones which became tolerant. However, if that were so, runting should never happen, owing to the constant overwhelming presence of host antigens. Furthermore it is clear from the present experiments that immunological facilitation protects against runting. If exhaustive sensitization were operative, the reverse would be expected since facilitation, by reducing the quantity of host antigens available to the sensitized cells, should decrease the process of exhaustive sensitization.

The explanation which seems the most likely to us is that active facilitation of the newborn by the homologous injected cells themselves is the basis for an answer to the problem under consideration. It seems now rather well established that immunologically competent cell populations are able to react in two immunological ways to homologous living cells (Snell, 1957b; Medawar, 1959; Mitchison and Dube, 1955): one way being the establishment of a state of delayed hypersensitivity towards transplantation antigens (Brent, Brown and Medawar, 1959) (which may be operative in the rejection reaction); the other way
being elaboration of circulating antibodies against antigens closely related to transplantation antigens (Gorer, 1957, 1958) (and known as enhancing antigens). These antibodies are able, when present at a certain level, to promote immunological facilitation or enhancement. The fate of the homologous injected cells or the homologous grafted tissues is likely to depend to some extent on the predominance of one of the two reactions, rejection or facilitation. But, although a predominant rejection reaction is a definitive phenomenon leading to the death of the graft, a predominant facilitation reaction, helping the graft to survive, is a precarious state that may be interrupted at any time. These considerations may help in understanding the events which follow an intravenous injection of immunologically competent cells into a homologous newborn mouse (Voisin, 1962): during several days the newborn is not immunologically competent; it provides only a nutrient medium and an antigenic stimulus to the injected cells. The immunological reaction of these cells is very likely to follow the general scheme of a two-headed reaction: rejection reaction and facilitation reaction; a competition can take place between these two reactions for rapidity of appearance and/or for intensity. If the rejection reaction of the injected cells toward the newborn predominates over the facilitation reaction, the animal will be runted and eventually die; if the facilitation reaction strongly predominates and/or precedes the rejection reaction, the animal will be protected and will survive—and this seems to be perfectly possible owing to the similarities between embryonic and tumour tissue (Hamperl, 1956; Bernhard, 1961); if the facilitation reaction predominates “weakly”, the animal will be partially or tardily runted. This interpretation is supported by the reported findings concerning runting and protection against it, particularly by the following facts: that runting, albeit being due to the same basic phenomenon as graft rejection, has not the same precise chronology, even using highly inbred strains of mice: it is often delayed or incomplete or does not exist at all.
These are precisely the three orders of results which can be experimentally obtained by passive enhancement of the newborn: retardation, attenuation or suppression of runting. Furthermore, the weight curves of the survivors of the two series of animals (protected and not protected) are extremely close, almost identical (except for a slight but constantly higher level of the curve for the protected animals) and, at the same time, they are much lower than the curve of the normal, non-injected control group. This is taken as a suggestion that the survivors, in the two groups, were submitted to analogous modifications, that is to say that the mechanism by which the survivors of the unprotected group overcame runting was probably analogous to the mechanism by which the treated animals were protected. Had the two mechanisms been different, one would have expected a difference to be observed some time during the evolution, either in weight or in another symptom. This proved not to be the case. It seems then both likely and reasonable to think that the facilitation reaction is the usual operative mechanism which protects newborns injected at birth with homologous cells against runting.

What then are the implications of this enhancing action of specific sera in newborn mice?

II. Immunological facilitation (enhancement phenomenon) applied to non-tumour tissues

A. Passive enhancement of non-tumour tissues

This work seems to be the first unequivocal passive transfer of immunological enhancement toward non-tumour tissue. In order to follow the development of the reactions and to visualize the identity between this experimental situation and the situation in enhancement of tumour homografts, one has to stop thinking in terms of donor and recipient and to think in terms of immunologically competent partner (the recipient of the tumour homograft or the splenic cells injected in the newborn) and immuno-
logically non-competent partner (the homografted tumour or the injected newborn). By doing so, one realizes at once that a newborn recipient (immunologically non-competent partner) can be passively "enhanced" or "facilitated" in such a way as to be protected against an otherwise lethal rejection reaction on the part of the injected cells (immunologically competent partner). This passive enhancement of non-tumour tissues has been shown to be unequivocal and sometimes dramatic, as it is with tumour tissues.

B. Reasons making this phenomenon possible

At this point, it is only possible to speculate upon the reason why embryonic or neonatal tissues react to immunological facilitation in a way closer to the reaction of tumour tissues than to that of normal adult tissues. In other words, why do neonatal tissues benefit from enhancement much more than normal adult tissues do? We are tempted to think that surface phenomena (including a high pinocytotic activity) common to tumour tissues, embryonic tissues (and also stem cells) might be the basis for a tentative explanation. If this were so one might predict that stem cells should also be susceptible to enhancement, a fact which would be very useful in the understanding of several phenomena related to transplantation immunity and acquired tolerance to living cells.

III. Acquired tolerance and its enhancement

The objective fact to be discussed is the significant increase in the proportion of tolerant animals and, above all, in the quality of tolerance, in the animals protected by facilitating serum as compared to non-protected animals. To try to explain this result would again require some knowledge of the nature and mechanism of immunological facilitation-enhancement. Unfortunately the operative mechanism is still poorly understood (probably as poorly understood as the action of Bogomoletz serum is!) and a
tentative explanation would lead to considerations which would be highly speculative.

Part of these will be detailed at the Prague symposium (Voisin, 1962). Possibly the most significant point is that the preceding results can hardly be accounted for by generally accepted views on immunological tolerance to living cells. There is an approach suggested by the first part of the reported experiments (on runting): passive facilitation of the newborn mice brings about something which is indistinguishable from a state of tolerance induced in homologous injected spleen cells toward the recipient. The only suggestion that we would like to make is that it might not be necessary to make that distinction. This state of tolerance is not necessarily a permanent one; the tolerance may be broken even after several weeks and the animal may die of runting disease: this seems to be the explanation why proportionally more passively protected animals died after grafting (between day 0 and day 99 after grafting) than did unprotected animals (16 out of 88, i.e. 18 per cent, versus 3 out of 38, i.e. 8 per cent). If facilitation leads actually to a state indistinguishable from tolerance, it might be of very great theoretical (and practical!) importance. This problem is at present under experimental study, but the results are not ready to be reported as yet.

**SUMMARY**

Each of 75 litters comprising 450 newborn mice was divided into three parts leading to three series. The first series (197 newborns) received adult CBA spleen cells plus immune serum prepared by immunizing adult CBA against either lyophilized newborn strain-A mice or against living strain-A tumour (according to a technique similar to the one used to obtain antibodies enhancing the growth of tumour tissues). The second series (170 newborns) received the same number of CBA spleen cells from the same preparation and the same amount of normal CBA serum.
The third series (88 animals) received nothing or physiological saline and were followed as normal controls. The observed results concerned runting syndrome and acquired tolerance to CBA skin grafts.

Concerning runting: between day 0 and day 35, 41 per cent of series 1 animals died of runting, 63 per cent in series 2 and 0 per cent in series 3; with powerful antisera the difference was still more striking (23.5 per cent death in series 1 versus 64 per cent in series 2). The weight curves of survivors of series 1 and 2 were almost superimposable and much below the weight curve of series 3.

Concerning tolerance: animals of series 1 exhibited a significant quantitative and qualitative increase in tolerance of CBA skin grafts. The immunological specificity of the antisera used was ascertained by specific haemagglutination and specific immunotoxicity.

Acknowledgements

We are very indebted to Dr. Francine Toullet, who helped us throughout this work mainly in performing haemagglutination tests. We are grateful to Miss F. Kelly who helped us in the statistical analysis.

REFERENCES


**DISCUSSION**

**Medawar:** Dr. Voisin, have you tried the effect of these antisera on the runting capabilities of presensitized CBA cells? If you compared presensitized with normal CBA cells it would help you to find out whether the antiserum was acting on the tissues of the A-line host and protecting them against the attack of CBA cells, or whether it was diminishing the immunological capabilities of the CBA cells themselves. These correspond to efferent and central inhibition.

**Voisin:** No, we have not tried that. As a matter of fact, this is a problem which is extremely interesting *per se*, but it was not the point we wanted to make in these experiments. It will be the subject of further experiments.

**Michie:** Does anybody know what runts die of—is it anaemia or liver damage?

**Voisin:** I do not know. And I doubt whether anyone knows exactly what is the ultimate cause of death in runting—or what is the ultimate cause of death in many other diseases. But the general mechanism of
ranting I think is no longer mysterious, although it might still yield surprises.

Billingham: After three years' hard work in collaboration with a pathologist, we have learned a lot of things about runt disease in the rat but we still don't know what kills them.

Nakic: I have had no experience with runt disease, but I have had with parabiotic disease which is just another form of graft-versus-host reaction. We have observed two forms of the disease: the "white" and the "red" form, the difference being that in the white form the predominant symptom is anaemia while in the other it is absent. In the white form one can be quite sure that the immediate cause of death is profound anaemia, which may develop in a matter of hours, the number of erythrocytes dropping from about 10 million to less than 2 million. I am not sure about the cause of death in the red form. One observes wasting, diarrhoea, focal necroses in the liver, and atrophy of the lymph organs. We haven't observed any overt infections, but nevertheless it may be an infection which finishes off the animal.

Russell: What is red in the red form?

Nakic: In parabiotic disease there is paralysis of the capillaries everywhere in the body, even in the animals suffering from anaemia. Therefore the non-anaemic animals appear very red because of the dilated capillaries while the grafts look pale by comparison although they are normally coloured.

Miller: What is the histological appearance of the thymus and the thyroid in animals suffering from runt disease?

Voisin: I don't know about the thymus in my animals. Dr. Billingham has done some work on this subject.

Billingham: In acute runt disease in the rat, there is complete atrophy of the thymus. Only a few strands of fibrous connective tissue can be located in the site normally occupied by this organ, and two nodes which are normally concealed by the thymus are very conspicuous in runts as a consequence of hypertrophy.

Krohn: This is typical of acute adrenal hyperactivity, which would go with the cause of death but probably not itself be the cause of death.
INDUCTION OF SPECIFIC TOLERANCE IN ADULT RATS BY THE METHOD OF PARABIOSIS*

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Institute of Physiology, Faculty of Medicine, University of Zagreb

The most important contribution to tissue transplantation research since Medawar’s demonstration (1944) that homograft rejection is an immunological phenomenon, was the discovery by Billingham, Brent and Medawar (1953) that the body’s immunological defence mechanism can be artificially overcome by inoculation of cells from an adult donor into immature recipients. In the same year Hašek (1953) described another experimental model known as “embryonic parabiosis” whereby artificial synchorial anastomoses were established between two bird embryos. Both methods were successful in inducing specific tolerance to subsequent skin homografts.

For some time it was believed that induction of tolerance to living tissue was limited by the so-called “adaptive period” which in most species ends around the time of birth. The first indication that it may not be so and that specific tolerance could be artificially induced in adult animals as well came from the radio-biologists. Main and Prehn showed (1955) that mice receiving a lethal dose of X-rays and protected with bone marrow from an F₁ hybrid derived from the irradiated and a foreign strain, will subsequently tolerate skin homografts from the foreign parent strain of the F₁ hybrid. Later on, many others showed that specific tolerance to homografts (Trentin, 1956; Barnes et al., 1958) or even heterografts (Zaalberg, Vos and van Bekkum, 1957) can be

* This work has been supported by grant no. 2919/2 from the Federal Scientific Fund (Belgrade).
induced in lethally X-irradiated adult animals treated with homologous or heterologous bone marrow respectively.

For the past six years we have been investigating the possibility of inducing specific tolerance to skin homografts in immunologically mature rats by the method of parabiosis.

**A short survey of results obtained in Y→W strain combination**

The attempts to maintain the parabiotic union in the Y→W strain combination to the 20th postoperative day or beyond proved unsuccessful (Nakić et al., 1961). Pairs were lost either through spontaneous separation of parabionts or because of the death of one of the partners as a result of "parabiosis intoxication" (see Finerty, 1952). Studies of the effect of the duration of the parabiotic union on the incidence of tolerance revealed that parabiosis of less than five days' duration was ineffective in producing tolerance of an appreciable degree. Best results were obtained with parabiosis lasting six days; prolongation of the parabiotic state to seven days or more did not bring about an improvement in the results. A possible explanation of this has been discussed elsewhere (Nakić et al., 1961).

Homografts exchanged between homologous rat parabionts separated after five to six days survive longer than in control animals (Nakić, Nakić and Silobrčić, 1960). The same is true when grafts from the third strain are transplanted into homologous parabionts, showing that this depression of the immune reaction might be non-specific. That the trauma involved in the parabiotic procedures is an unlikely cause of this phenomenon has been shown by the absence of prolonged homograft acceptance if genetically related parabiotic partners are grafted with homologous skin transplants (Nakić and Silobrčić, 1962).

The majority of separated parabionts that failed to destroy the cross-graft fell ill and died of an illness which we had named
“parabiotic disease” (Nakić and Silobrčić, 1958). Tested in extremis, these parabionts were found to be chimeras (Nakić and Silobrčić, 1962).

The characteristic symptoms of “parabiotic disease” were wasting, diarrhoea, anaemia and general lymphoid atrophy in an animal tolerant of the skin graft from its parabiotic partner. These symptoms were never observed in a parabiont that had successfully rejected the cross-graft. This close relationship of tolerance to the disease was absent in parabionts challenged with the graft from the third strain (Nakić and Silobrčić, 1962). Symptoms of the disease appeared several days after complete breakdown of the graft; even when parabionts fell ill with a graft that was still preserved, the symptoms continued for several days following rejection of the graft. Thus it appeared that once the symptoms of “parabiotic disease” had set in, inhibition of the immune reaction became specific, the sick animal being quite capable of differentiating between the cross-graft and the graft from the third party.

Because of the close relationship of the disease to tolerance and the striking similarity between “parabiotic disease” and “parabiosis intoxication” on the one hand and conditions such as “runt disease” and “secondary disease” on the other, we have included “parabiotic disease” or “parabiosis intoxication” in the now large family of graft-versus-host reactions (Nakić and Silobrčić, 1958). Since it seems that the term “parabiosis intoxication” may cover several phenomena (Eichwald et al., 1961) we suggest that the term “parabiotic disease” be reserved to denote the graft-versus-host reaction in parabionts.

The susceptibility to “parabiotic disease” has been found to fall sharply in parabionts two months of age or older (Nakić et al., 1961). Younger hosts, unable to reject the cross-graft, all succumb to “parabiotic disease”. The relation of age to susceptibility to graft-versus-host reaction has already been described by Cock and Simonsen in chicks (1958) and byBillingham and
Brent in mice (1959). Older parabionts may display a high degree of tolerance without fatal manifestations of “parabiotic disease”. Some hosts may show mild recurrent attacks of the disease alternating with immune crises in the graft.

**The hypothesis of competitive replacement**

Based on the results described above, the competitive replacement of the immune system of the host by that of the donor has been proposed as a possible mechanism responsible for induction of tolerance following short-term parabiosis between bilaterally incompatible partners (Nakić et al., 1961). The replacement would take place as a result of transfer of immunologically competent cells from one parabiont to another through vascular anastomotic channels. The ensuing competition between actively immunized cells of the host and the donor would damage the lymphopoietic tissues of the host and cause non-specific depression of the immune reaction. In combinations where antigenic conditions favour a stronger unidirectional immunity reaction the more reactive partner may, by an immunological vicious circle, exterminate host lymphoid tissue and replace it with its own. Specific tolerance would result, the immune system of the donor “tolerating” grafts of donor type and reacting against any other antigen. “Parabiotic disease” would thus be a manifestation of the immunological attack of donor cells against the host.

**Induction of tolerance in Wistar→Y59 strain combination**

Our more recent work concerns another strain combination, Wistar→Y59. Wistar rats were obtained from another Institute* where inbreeding of this non-homogeneous stock has been started. The offspring of the F₄ generation have been tested by us and permanent survival of mutually exchanged grafts was found

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* Institute “Rugjer Boškovic”, by courtesy of Prof. N. Allegretti.
in 45 per cent of recipients. The Y59 strain is being developed from the Y stock used in our previous experiments. The results of some recently conducted grafting tests showed that grafts on over 80 per cent of hosts were still in good condition after 100 days.

The technique of skin grafting and the parabiosis operation have been described elsewhere (Nakić et al., 1961; Nakić and Silobrčić, 1962). The normal mean survival time (± standard deviations) of Wistar grafts in Y59 recipients is $8.4 \pm 1.1$ days

**Table I**

**Survival times of homografts exchanged between Y59 and Wistar parabionts**

<table>
<thead>
<tr>
<th>Parabiont</th>
<th>Survival times of homografts (days)</th>
<th>Mean expectation of life (± standard deviation)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>9, 9 × 10, 12 × 11, 5 × 12, 3 × 13</td>
<td>$11.0 \pm 1.0$</td>
</tr>
<tr>
<td>Y59</td>
<td>${ 8, 2 \times 9, 3 \times 10, 3 \times 11, 2 \times 12, 3 \times 13, 2 \times 14, 16, 21, 23, 28, 33, 47, 48, 65, 73, 78, 4 \times &gt; 100 }$</td>
<td>$&gt; 34$</td>
</tr>
</tbody>
</table>

* Operation for parabiosis and cross-grafting performed simultaneously; parabionts were separated after six days.
† Normal average survival times (± standard deviations) of skin grafts transplanted from Y59 to Wistar rats and *vice versa* are as follows:

- Y59→Wistar: $9.6 \pm 1.2$ days
- Wistar→Y59: $8.4 \pm 1.1$ days

and that of Y59 grafts in Wistar hosts $9.6 \pm 1.2$ days. Adult rats of both sexes were used. Parabionts were separated after six days. The results are presented in Table I.

As can be seen, prolonged survival of grafts was induced in parabionts from both strains, but the degree of tolerance conferred upon Wistar parabionts was very low, the breakdown of all grafts having been completed by the 13th postoperative day. Such asymmetry in the tolerance responsiveness between Wistar and Y59 parabionts was not noted in the Y59→W combination*

* The W strain is genetically unrelated to Wistar rats and strains derived therefrom.
where the number of tolerant animals and degree of tolerance were about even in the two strains. We believe that this difference is due to the greater genetic homogeneity of the strains used in the present series.

No clinical symptoms of "parabiotic disease" have been observed in any of the tolerant Y59 parabionts. This is an interesting finding in view of the high susceptibility of Y59 animals to "parabiotic disease" in combination with W partners. However, all the tolerant animals displayed at least some degree of lymphoid atrophy as found by histological analysis of their spleens and lymph nodes.

Although the immune reaction in most grafts carried by tolerant animals was just beginning at the time when grafts in control recipients would have already shown an advanced degree of epidermal breakdown, superficial scaling or multiple small necrotic areas were observed in all recipients by the end of the second week. These necroses either spread to involve the entire graft or the process of destruction became arrested for a while only, to be resumed after a few days. Such recurrent attacks continued until complete destruction of most grafts took place. In hosts displaying long-term tolerance, the immune reaction slackened considerably during the fourth postoperative week. As a result of this, the condition of the graft became rather stable, but by that time most grafts were considerably reduced in size and of some only a vestige remained. It was this apparent cessation of the immune reaction that prompted us to test both the degree and the specificity of tolerance.

**The specificity and degree of tolerance induced in Y59 parabionts**

Table II shows results of the experiment in which seven tolerant parabionts were challenged with the second cross-graft and a graft from a foreign strain unrelated to either parabiont.
The test was performed four to eight weeks following the operation for parabiosis and cross-grafting and at least two weeks following the last observed immune crisis in the graft. The foreign strain used was AGA black rat obtained from a mutant of a cross\* between wild gray male and albino female and now in the F_7 generation. Destruction of skin homografts exchanged between members of the AGA and Y59 strains is invariably complete within 12 days.

As shown in Table II, all AGA grafts were destroyed by the 14th postoperative day, while second grafts from parabiotic partners were well accepted. There was a good growth of hair, and both the first and second cross-grafts grew in size as the animals gained weight. At the time of rejection of the AGA grafts several punctate necroses were observed in both cross-grafts but healed quickly. Breakdown of tolerance was marked by practically simultaneous rejection of both cross-grafts.

It is interesting to note the delay with which AGA grafts were

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\* Obtained from the Institute “Boris Kidrič”, Vinča, Belgrade, by courtesy of Dr. P. N. Martinović.
disposed of by the tolerant animals; no graft has in fact been rejected before the 10th postoperative day, which approximately represents normal mean survival time of AGA grafts in Y59 hosts. A similar “lag” has also been observed in Y59 and W recipients suffering from “parabiotic disease” and challenged with Wistar skin grafts (Nakić and Silobrčić, 1962). A subnormal immune response by the homologous radiation chimeras to skin grafts from a strain not involved in the chimeric constitution has been reported by Doak and Koller (1961) and to sheep red blood cells by Doria and co-workers (1962). This phenomenon is similar to that already reported by Billingham and Brent (1959) regarding the impairment of a tolerant mouse’s ability to reject skin homografts from a strain unrelated to either donor or recipient. A possible explanation is given elsewhere (Nakić, 1962).

The immune status of Y59 parabionts following breakdown of tolerance

In order to determine the immune status of tolerant parabionts following breakdown of tolerance, ten such parabionts received test grafts from parabiotic partners three to four weeks after complete destruction of cross-graft(s). The results summarized in Table III show that following low-degree tolerance the animal remains in the state of heightened resistance and the next graft from the parabiotic partners is rejected as a “white graft”. Parabionts which disposed of their grafts during the fourth postoperative week or later behave differently. In these, the breakdown of tolerance is followed by a “null period” lasting several weeks during which the formerly tolerant animal disposes of the second graft from the parabiotic partner by the first-set reaction. Even the third cross-graft placed two weeks after rejection of the second cross-graft may fail to evoke a typical immune reaction (“white graft”).
### Table III

**Immunological status of Y59 parabionts following breakdown of tolerance**

<table>
<thead>
<tr>
<th>Breakdown of tolerance* (days post-operative)</th>
<th>Appearance of first test-graft† (5 days after grafting)</th>
<th>Survival times of first test-graft (days)</th>
<th>Second test-graft‡ (with survival time in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>White</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>White</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>White</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>White</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Normal§</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>White</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Normal</td>
<td>10</td>
<td>Pink but poorly healed-in</td>
</tr>
<tr>
<td>48</td>
<td>Normal</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Normal</td>
<td>8</td>
<td>White (7)</td>
</tr>
</tbody>
</table>

* Day of complete rejection of cross-graft(s).  
† Test-grafted 3–4 weeks following breakdown of tolerance.  
‡ Placed two weeks after rejection of first test-graft.  
§ As judged by colour, adherence to graft bed and survival time.  
|| Torn off by host on 4th postoperative day.

### Attempts to increase the incidence of tolerance in Y59 parabionts by preimmunization of Wistar rats

Wistar rats were preimmunized with spleen cell suspension from Y59 donors. Five days later donors were joined in parabiotic union with the immunized recipients and cross-grafts exchanged. The parabionts were separated after six days.

The purpose of the experiment was to give Wistar parabiotic partners an artificial advantage whereby an increase in the incidence of tolerance would be gained. As can be seen from Table IV, no such increase has been obtained but four animals developed symptoms of “parabiotic disease” and died with well preserved grafts. Anaemia was absent in these cases and the sick animals appeared red because of the general capillary dilatation; the cross-graft although normally coloured looked pale by comparison. This “red form” of the disease ran a less acute course than the
'white form' observed in a previously used combination where anaemia was the prevalent symptom.

Despite the presensitization none of the Y59 grafts in Wistar parabionts was rejected by the second-set reaction ('white graft') and the mean survival time of grafts was found even to be somewhat prolonged (10.8 ± 1.7 days). That the pretreatment of

Table IV

<table>
<thead>
<tr>
<th>Parabiont</th>
<th>Survival times of homografts (days)</th>
<th>Mean expectation of life (± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>7, 9, 5×10, 3×11, 3×12, 13, 14</td>
<td>10.8 ± 1.7</td>
</tr>
<tr>
<td>Y59</td>
<td>{8, 9, 2×10, 2×11, 12, 13, 14, 16}</td>
<td>{&gt;18†, 18, &gt;31†, &gt;33†, &gt;41†}</td>
</tr>
</tbody>
</table>

* Immunization by i.p. injection of spleen cell suspension from Y59 donors five days before operation for parabiosis and cross-grafting.
† Died of 'parabiotic disease' with surviving cross-graft.

Wistar rats has, in spite of the above finding, been effective in sensitizing the recipients is revealed by the induction of fatal "parabiotic disease" in a strain combination where it normally is not induced. Preliminary experiments indicate that the masking of the immune state in Wistar parabionts might have been caused by parabiotic trauma, although we have failed to show the effect of parabiotic trauma on survival time of first-set grafts (Nakić and Silobrčić, 1962).

**Chimerism**

Tolerant parabionts were tested for chimerism by the method based on Mitchison's design as described by Billingham and Brent (1959). Secondary hosts first received i.p. spleen or lymph-node cell suspension from the tolerant parabiont and four to five days later a skin graft from the parabiotic partner of the tolerant animal. The presence of donor cells is shown by the finding of
the "white graft" phenomenon in the secondary hosts. Less than \(1 \times 10^6\) spleen or lymph node cells is sufficient to give a positive reaction.

All the tolerant animals tested by the above method were found to be chimeras. An interesting fact emerged from these studies. Though the parabionts were found to be chimeras as long as they were tolerant, some of the animals that succeeded in rejecting the cross-graft after a period of tolerance of at least two weeks, were also found to be chimeras irrespective of whether the test graft transplanted at the time when the test for chimerism was performed, was rejected by the first- or the second-set reaction.

Although the above test is an excellent one for the qualitative demonstration of foreign cells, we found it necessary to devise a test that could render possible both the qualitative and the quantitative demonstration of donor cells in any rat strain. The test that could best fulfil these qualifications was obviously a cytological one and the possibility of using sex chromosomes as "markers" was provided by genetic studies of S. Ohno and co-workers (Ohno and Kinosita, 1955; Ohno, Kaplan and Kinosita, 1958; Ohno, Kaplan and Kinosita, 1959). By joining in parabiosis partners of opposite sex it was possible by this method to identify male cells in a tolerant female parabiont and *vice versa*.

The only way in which sex can be decided in mitotic metaphases of rat somatic cells (Ohno and Trujillo, personal communication) is by photographing the cell, then cutting out the chromosomes, pairing them and aligning them serially. In female somatic cells there will be an additional pair of chromosomes similar to the second, third and fourth largest pair of autosomes; this is the X pair. In male somatic cells, after pairing has been completed there will be one left-over chromosome similar to the second, third and fourth largest autosomes, which is the X-chromosome, and another left-over similar in size to the smallest acrocentric pairs, which is the Y-chromosome. Fig. 1 shows a photomicrograph and an ideogram of a male cell recovered from the bone
Fig. 1. Photomicrograph (a) and ideogram (b) of a male cell recovered from the bone marrow of a tolerant female parabiont dying of parabiotic disease 33 days following the operation (identified by courtesy of Dr. Y. Trujillo, City of Hope Medical Center, Duarte, Calif.).
marrow of a tolerant female parabiont dying of “parabiotic disease” 33 days following the operation.

The cytological method may well be applicable in other mammalian species, including man, and a similar method has already been used in the chicken by Biggs and Payne (1959).

**Conclusions**

The availability of a strain combination in which the phenomenon of tolerance is not associated with clinically manifest graft-versus-host reaction renders possible a more accurate observation of the successive stages that lead to the establishment of the state of specific tolerance in adult parabionts.

The apparent paradox of tolerance following short-term parabiosis is that the state of specific non-reactivity is induced in an immunologically competent animal by a procedure that primarily causes sensitization. In some parabionts the immune reaction is of sufficient intensity as to cause destruction of the skin homograft by the 12th postoperative day. In others, a more lingering process may achieve the same result, that is total destruction of the cross-graft, by a series of successive immune crises. In still others, the immune process becomes seemingly arrested during the fourth postoperative week but not before the cross-graft has been considerably reduced in size. In these animals the next cross-graft is well accepted, sometimes throughout life.

What then, is the mechanism by which an already sensitized animal is subsequently rendered specifically unresponsive? We had already suggested (Nakić et al., 1961) that the basic factor responsible for this phenomenon might be the difference in immunological reactivity between the parabiotic partners. This factor would determine the difference in rate and intensity by which the process of immunization would proceed in either parabiont despite the fact that both are antigenically stimulated at the same time. The asymmetry in tolerance responsiveness
observed in the strain combination used in the present series of experiments indicates that the difference in reactivity might be under strict genetic control. This is further shown by the fact that preimmunization of Wistar parabionts does not result in increased incidence of tolerance in Y59 partners. It seems that the only advantage possessed by the preimmunized animal remains the one inherent in the genetic set-up of the relative strain combination. Previous immunization may only accentuate the difference in reactivity between the partners either as regards the number or quality of immunized cells. This may be the answer to the question why cells from a preimmunized donor can induce “parabiotic disease” in a strain combination where cells from a non-preimmunized donor cannot.

More precisely, a situation favouring a stronger immunity reaction in one direction than in the other would obtain in combinations where the number of strong antigens possessed by one partner exceeds that of the other partner. In a very simplified form, it could be presented in the following way:

\[
x: A\ B\ -\ D\ E\ F\ G
\]

\[
y: A\ -\ C\ D\ -\ -\ G
\]

where partner “x” lacks C antigen but possesses BEF antigens not present in partner “y”. In this donor-host bilaterally incompatible combination one could expect that partner “y” would develop a stronger immunity reaction against partner “x” than would be the case the other way round. Thus, when vascular anastomoses are established between parabionts around the fourth postoperative day, the number of blood-borne cells exchanged may be the same, but the number of immunologically active cells will be different, the more slowly reacting partner receiving a greater number. The greater the damage inflicted on the lymphopoietic system of the recipient by the transferred donor cells the less will be its capacity to react. This immunological
vicious circle may lead to the extermination of host lymphoid tissue and replacement thereof by donor cells. By the above process gradual "desensitization" of the host may take place with the concurrent establishment of specific tolerance.

However enticing the idea of complete replacement of the immune system of the host had at first appeared, it soon became evident that replacement in the anatomical sense of the word was a goal very difficult to attain if attainable at all. That a tolerant chimera may contain immunologically active cells of both host and donor origin is revealed by alternating immune crises described elsewhere (Nakić et al., 1961); a tolerant parabiont may recover from "parabiotic disease" with the graft soon showing necrotic patches—a host-versus-graft reaction. If regeneration of the graft takes place, the host may again show symptoms of "parabiotic disease". Such alternating crises may frequently recur. It would seem that tolerance is not a static phenomenon but would be maintained by a very dynamic balance struck between host and donor cells. A favourable donor/host cell ratio might maintain tolerance indefinitely. A shift in balance towards the host side would cause abolition of tolerance while a decrease in the activity of host cells would result in fatal graft-versus-host reaction.

**Summary**

Short-term parabiosis (5–6 days) between adult bilaterally incompatible albino rats is followed by non-specific inhibition of immune reaction which may gradually progress to specific tolerance.

In one strain combination, tolerance is associated with "parabiotic disease", a fatal condition considered to be a graft-versus-host reaction. In another strain combination, specific tolerance can be induced without manifest signs of parabiotic disease unless one of the partners is preimmunized. The first cross-graft may be partially rejected but the second graft from the same donor one to
two months later is well tolerated, although a simultaneously transplanted graft from the third party is destroyed.

The breakdown of tolerance is followed by a "null period" during which the formerly tolerant animal disposes of the second graft from the parabiotic partner by the first-set reaction.

Tolerant parabionts are chimeras. A new cytological method for identification of donor cells in tolerant chimeras is introduced.

The competitive replacement of the immune system of the host by that of the donor has been proposed as a possible mechanism responsible for induction of tolerance following short-term parabiosis.

Acknowledgements

We are perpetually in debt to Dr. S. Ohno, Dr. J. Trujillo and Mrs. Patricia A. Ray of the Department of Experimental Pathology, City of Hope Medical Center, Duarte, California for their continued help and advice. We are also grateful to Mrs. Gjurgja Horvat for her assistance in this work.

REFERENCES


Medawar: I take it, Dr. Nakic, that your theory of tolerance implies that you agree with Dr. Voisin that tolerance of living cellular antigens is a totally different phenomenon from tolerance of “dead” antigens such as soluble protein?

Nakic: That is correct.

Voisin: It might be more accurate to say that tolerance of living cells is a phenomenon different from and more complex than tolerance of chemically defined antigens. In this respect, Dr. Nakic made a very important point, that is, the presence of immunized cells from both host and donor strain in the spleen of tolerant animals. If these results are confirmed, they will have extremely important implications. As a matter of fact, they are already confirmed in a sense, for we have obtained the same kind of result as Dr. Nakic with a completely different experimental set-up (Leonard, L., Kinsky, R. and Voisin, G. American Association for Advancement of Sciences. Biology section. Arizona Academy of Science. April 1961). We started with a weakly incompatible strain combination, BALB/c→DBA/2, injecting homologous spleen cells into newborns, and did not produce runt disease with doses of about 7 million cells. The only way to get runt disease is either to inject a very large dose of homologous cells (and this is technically very difficult) or to use preimmunized cells. But, now, if we take spleen cells from a BALB/c mouse tolerant to DBA/2 and inject them into newborn of either BALB/c or DBA/2 strains, then we can observe a certain percentage of runting which is around 10 per cent of
lethal runting, with almost half of the injected animals having a decrease in their weight curve, while animals injected with normal isologous cells have no decrease in weight. It seems difficult to escape the conclusion that under these experimental conditions the spleen cell population of a tolerant animal reacts in a way which is closer to the way in which immunized spleen cells react than to the way normal spleen cells react. And this seems to me to be extremely important, especially in view of our working hypothesis of recent years which is concerned with the relationships between specific acquired tolerance to living cells, runt disease and enhancement phenomenon.

This working hypothesis is diagrammatically represented in Fig. 1. When an injection of immunologically competent cells is made intravenously into a homologous newborn mouse, the subsequent immunological phenomena will evolve in two consecutive phases depending on the state of immunological maturation of the young animal.

Fig. 1 (Voisin). Diagrammatic representation of the proposed hypothesis. General scheme: the intensity of the respective reactions (arrows) will lead to the possibilities represented in the following figures.
During the first phase the newborn is not immunologically competent; it provides only a nutrient medium and an antigenic stimulus to the immunologically competent injected cells. In turn, these cells react against the host and they can do so in two ways, leading respectively to a rejection reaction and a facilitation reaction. A competition takes place between these two reactions for rapidity of appearance and/or intensity.

Fig. 2 (Voisin). Phase I (cells versus host). Predominance of the rejection reaction over the facilitation reaction, leading to runting.

During the second phase, that competition can continue, but now the young animal becomes immunologically competent and starts to respond to the antigenic stimulus of the injected homologous cells; this immunological response can also be exerted in two ways—rejection and facilitation. Here again a competition takes place.

The direct logical consequences of the hypothesis are rather obvious. During phase I, if the rejection reaction of the injected cells towards the homologous newborn predominates over the facilitation reaction, the animal will be runted and eventually die (Fig. 2); if the facilitation
reaction predominates strongly and/or precedes the rejection reaction, the animal will be protected and will survive (Fig. 3); if the facilitation reaction predominates "weakly", the animal will be partially or tardily runted.

If the animal survives without apparent runting or with a moderate runting, phase II will take place.

During phase II, if the rejection reaction of the young mouse toward the homologous cells predominates, the animal will reject the cells and will not be tolerant (Fig. 4). If the facilitation reaction predominates and/or precedes the rejection reaction, the cells will be protected and survive (Fig. 5): a state of active immunological equilibrium will be reached, which will possibly include a weak reciprocal rejection reaction. But the important thing is that this rejection reaction, if it exists, will be preceded and continuously counteracted by a strong reciprocal facilitation reaction, and the animal can then be considered as tolerant to the injected cells and other cells of the same strain which
DISCUSSION

will further benefit by the same immunologically balanced reactions. Various factors, among which the strain combination is certainly the most important, will determine which type of reaction will predominate at phase I and at phase II.

The logical consequences of this concept (namely the possibility of a homologous injection of spleen cells into a newborn mouse resulting

![Diagram]

Fig. 4 (Voisin). Phase II (host versus cells). Predominance of the rejection reaction over the facilitation reaction: cells are rejected; the mouse is not tolerant.

either in homologous disease or in immunological tolerance, or in no obvious modification) precisely coincide with what is actually observed in current experimentation.

It must be understood that phase I is not a prerequisite for phase II to take place. If one succeeded in injecting adult living, surviving, exclusively immunologically incompetent cells into a newborn animal, the immunological story would start directly at phase II with immunological reaction only on the part of the host, thus without any risk of runting. On the other hand, if one injects spleen or liver cells from a
homologous foetus into a foetus or a newborn animal, phase I and phase II will start at the same time (sometime around the second week in mice); in that situation, the host will be in a better position and a reciprocal enhancement would be the most likely consequence. It must also be understood that this concept does not automatically preclude other immunological phenomena from taking place.

Finally, the main feature of the proposed hypothesis resides in that, instead of considering immunological tolerance to living cells as a passive state of immunological non-reactivity induced in immature animals, it is considered as an active state of a two-way immunological reactivity resulting in a delicate balance.

Woodruff: We (Michie, D., Woodruff, M. F. A. and Zeiss, I. [1961]. Immunology, 4, 413) have some experiments which point to conclusions very different from those of Nakić about this takeover business. In adult A mice which were made tolerant of CBA by an injection of spleen cells at birth, we find that most of the immunologically com-
petent cells in the spleen are A-strain, and only a small proportion are CBA. On the other hand, spleen cells from this chimera, unlike spleen cells from a normal A-strain mouse, do not cause splenomegaly when injected into newborn CBA mice. This investigation was prompted by a question I asked at the meeting in Liège in 1959: are there such things as tolerant cells? I wouldn’t say this experiment proves there are tolerant cells, but it does prove that the concept of tolerance applies to a population of cells at any rate.

Nakić: Lacking the exact quantitative analysis of the chimeric state in tolerant animals it is rather difficult to draw these conclusions.

Silvers: After parabiosis, when the immunologically competent system of one partner has apparently been replaced by that of the other, have you ever challenged the animals with autografts, and are they accepted? Dr. P. Koller and his associates have reported that homologous, radiation-induced mouse chimeras can, indeed, reject their own skin and we have also found this to be the case in similar experimental situations.

Nakić: We have intended to do that, but we haven’t done it.

Medawar: Dr. Nakić, could we just be clear on one point: does your theory imply that adult cells which are not immunologically competent couldn’t produce tolerance?

Nakić: This is a rather delicate question. From the viewpoint of the concept of the competitive replacement I would prefer that they could not, but evidence has been presented that tolerance can be induced with F₁ hybrid cells—so either my theory is wrong or there are several distinct phenomena, all fundamentally different, but all contributing to the induction of tolerance to living tissue.
MODIFICATION OF RUNT DISEASE IN MICE BY VARIOUS MEANS

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Studies of the course of events in a newborn recipient mouse following the injection of a population of lymphoid cells make possible an assessment not only of the immunological capacity of the inoculum by its ability to produce runt disease but also of its persistence at full antigenic capacity, most critically assessed by its ability to confer specific immunological tolerance. The latter is not possible in systems using "genetic tolerance" of the recipient rather than immaturity to ensure a hospitable environment to the injected cells. Thus, where adult parental strain cells are administered to adult F1 hybrids, for example, the convenient skin graft test is not helpful in demonstrating the persistence of the injected cells which are lost amongst neighbouring cells bearing identical antigens.

Simonsen has described and used the phenomenon of splenic enlargement in the recipient most effectively in the quantitative assessment of the severity of the reaction of adult lymphoid cells against hosts of varying age and has brought this technique to a stage of considerable refinement (Simonsen, 1957; Simonsen and Jensen, 1959; Simonsen et al., 1958). The fact that serial observations cannot be made on the same animal makes this method less attractive as a means of following the onward course of the disease in small groups of animals. Although one might be able to follow quantitatively other features of this complex disease (see Howard, 1961), the newborn mouse is a small target for analysis and we
have accordingly elected to use the simple quantity of total body weight as an overall estimate of the progress being made by the diseased animal. This criterion is, of course, readily supplemented by observations of the incidence of disease and the time of death where applicable. A preliminary report of our use of the weight-gain method has previously appeared (Russell, 1960) and is amplified by the present communication. This method has also been used by Anderson, Delorme and Woodruff (1960).

Plan of experiments

The experiments were divided into three parts. First, the course of runt disease as exhibited by the present strain combination of mice, DBA/1 donors and C57BL/6 recipients, was determined along with some of its pathological features. The importance of the dose of foreign cells injected was investigated and some information was gained as to the effect of delivering the injection by different routes.

Secondly, efforts were made to influence the otherwise virtually inevitable course of the disease by methods designed to specifically inactivate or destroy the foreign lymphoid cell inoculum. Two types of treatment were used here:

(1) A second injection of spleen cells was given from adult donors isogenic with the neonatal recipients, administered either by mixing the isologous cells with the homologous cell suspension before injection, by separate administration immediately after the homologous spleen cells via another vein, or by delaying the second injection for varying periods after the first. Second injections of cells from animals previously sensitized to DBA/1 tissue by skin grafting were also used.

(2) Secondary injections of serum were given from animals sensitized to the original donor strain (DBA/1) either by skin grafting or by injections of spleen cells with an adjuvant mixture in the foot pads, as detailed below.
Thirdly, several treatments of a non-specific type which might be expected to interfere with the immunological reactivity of the injected lymphoid cells were tested. These were as follows:

1. a series of injections of cortisone acetate (0.025 mg./g. body weight);

2. a single exposure to whole-body X-irradiation two to five days after the spleen cell injection.

A short course of treatment with each of two antimetabolic drugs has also been tried.

3. Burroughs-Wellcome 57–322* was selected as one of a group of the drugs which act on nucleic acid metabolism by competitive inhibition of purines.

4. Amethopterin (methotrexate) was chosen as an example of an antifolic acid compound.

Finally, a few preliminary attempts to achieve the secondary transfer or passage of runt disease have been made. As stated above, almost all experiments have included the dual purpose of evaluating not only the impact of the injected cells upon their recipients but also, whenever the animals lived, of testing the specific effect of the foreign cells, under the conditions of the particular experiment, on the host’s ability to react to tissues of donor origin by observing the behaviour of a graft of donor strain skin.

Materials and Methods

Animals

Mice of highly inbred strains were used throughout. C57BL/6 neonatal animals were employed as recipients and DBA/1 adults of both sexes as spleen cells donors.† These strains differ

* Generously supplied by Dr. George H. Hitchings of Burroughs Wellcome Co. (U.S.A.) Inc.
† Both strains were obtained from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine, and were maintained by a system of pedigreed matings as inbred stock in such a way as to minimize the development of separate sublines within the colony.
genetically at the H-2 locus, C57BL/6 being classed as H-2^b and DBA/1 as H-2^g. This strain combination thus provides major histocompatibility differences in both directions (Amos, 1959).

When obviously pregnant the mother of each prospective litter was placed in a separate cage where she was kept with her young for the remainder of the experiment.

**Cell suspensions**

The donor spleens were promptly removed from adult DBA/1 mice with aseptic precautions immediately after sacrifice by cervical dislocation. One to two spleens usually provided a sufficient number of dissociated cells free from connective tissue for injecting a single litter since a harvest of about 500,000 usable nucleated cells per mg. of spleen was obtained. The spleens were pooled in a sterile Petri dish, cleaned of surrounding connective tissue, cut into small pieces with fine scissors, and gently pressed once through a stainless steel sieve (250 holes/cm.²), washing liberally with a balanced tissue culture medium (TC-199)* as described by Billingham, Brent and Medawar (1956).

The cell clumps were broken up by gentle flushing through a fairly coarse pipette and were concentrated in a plug by centrifuging at about 500 g for five minutes. The cells were then washed twice more by resuspension in 6 to 8 ml. of TC-199 and centrifuged as before, thus avoiding the deaths in acute apnoea which have been known to follow the injection of appreciable quantities of the fragments of broken cells (Billingham and Brent, 1959). No anticoagulant was used. The final volume was adjusted so that the prescribed number of nucleated cells, as determined by haemocytometer counts, could be delivered in a volume of 0.04 to 0.07 ml.

* TC-199 available from Microbiological Associates, Bethesda, Maryland.
Intravenous injections

Except where otherwise noted all injections were performed through the facial or submental vein of the newborn mouse using a no. 30 gauge needle according to the method of Billingham and Brent (1957). No more than twelve hours were allowed to elapse between birth and the initial spleen cell injection. Animals dying within 48 hours after injection were assumed to have succumbed to its direct mechanical consequences and were excluded from further consideration.

Skin grafting and sensitization

In those experiments requiring spleen cells from donors previously sensitized to the tissues of another mouse strain this was accomplished by either of two different methods. The usual method consisted merely of applying full-thickness skin homografts bilaterally to bare areas prepared on the thoracic wall as described by Billingham and Medawar (1951). The prolonged state of sensitivity produced by rejection of such fixed tissue grafts was usually reinforced in the animal not more than six days before its use by an intraperitoneal injection of about 10 million dissociated spleen cells derived from an adult member of the same donor strain.

The other method of sensitization made use of suspensions of donor spleen cells exclusively as the antigen. The cell suspension, prepared as described above, was mixed to form an emulsion in equal volumes with a complete adjuvant mixture.* About ten million cells were injected into each of the four foot pads of an anaesthetized adult recipient on two different occasions seven days apart. Five to nine days after the second dose of cells in adjuvant, blood was collected by cardiac puncture and the serum separated for use. The serum was used promptly without

* Paraffin oil (Bayol F, Esso Standard Oil Co.), 8·5 ml.; mannide monooleate (Arlacel A, Atlas Powder Co.), 1·5 ml.; heat-killed human tubercle bacilli, 40 mg.
storage. Where test skin grafts were performed they were done in the manner referred to above.

Weights
The weight of each neonatal animal was measured to the nearest 0.01 g. on the first or second day of life and generally every two to three days thereafter throughout the period of the experiment.

Histology
Microscopic examination of tissues has been confined to standard preparations made after fixation in 10 per cent formalin or Zenker's formol, by paraffin embedding, sectioning at 8μ and staining with alum haematoxylin and eosin.

Results
I. Runt disease, general features
(1) Time course of development and pathology. Normal neonatal C57BL/6 mice begin life with a period of rapid gain in total body weight which is fairly linear over the first 30 days (Fig. 1a). Although this curve of normal growth is reliable enough to be used as a background for comparison of gross changes, controls within a single litter have been used whenever possible. Following the intravenous administration of 5 to 10 million DBA/1 spleen cells, newborn C57BL/6 mice follow a normal course of weight gain until the sixth or seventh day. At this time (see Fig. 1b) there is a sudden cessation of weight gain. The animals enter upon a "plateau period" and soon thereafter begin to die. Of 140 neonatal recipients receiving about 5 to 15 million foreign spleen cells intravenously on the first day of life all but 6 (3.7 per cent) died of the effects. The median age at death was 14 days (Fig. 2). Three of these survivors were later grafted with DBA skin which was fully rejected by 11 days indicating no
significant alteration in reactivity from the normal to tissues of the donor strain.

Since most of the animals die promptly and without reaching a body weight of more than about 3 or 4 g, the gross appearance of the animals is not as strikingly abnormal with this combination of

strains as with some others (Billingham, 1958). Acutely ill animals show some loss of hair and a slight scaling of the skin, a change which becomes more obvious in those which live a little longer (Fig. 3). The abdomen is somewhat protuberant and a few mice suffer from diarrhoea. Examination of the abdominal contents of animals killed when moribund often reveals small collections of
Fig. 3. An animal suffering from advanced runt disease shortly before death. This animal had received 12 million homologous spleen cells on the day of birth and had lived for 21 days, longer than usual. Note the scaling of the skin, protuberant abdomen and profound stunting.

(Approx. × 2.)
Fig. 4. Liver from runt killed at 2 weeks showing area of subcapsular necrosis with some surrounding areas of parenchymal cellular infiltration. The cellular infiltrate consists mainly of mononuclear cells although some polymorphonuclears are present. (Haematoxylin and eosin. × 66.)
ascitic fluid and moderately enlarged livers with sharply defined yellow patches along the free margins of the lobes, a finding also mentioned by Siskind and Thomas (1959). Histological examination of the liver shows these areas to consist of coagulative necrosis associated with a varying amount of widespread patchy cellular infiltrations, most notable in the periportal areas and including both lymphoid cells and polymorphonuclear leucocytes (Fig. 4). Since the haemopoietic elements present in the liver at birth do not normally disappear for some days thereafter, the interpretation of the origin of such cells in very young animals may be particularly difficult.

The spleen is usually firmer than normal and somewhat enlarged, although this change is also more marked in animals which have lived longer, presumably with a less acute form of the
disease. Distinct Malpighian corpuscles are rare on microscopic examination as the normal splenic architecture gives way to a population of large pale histiocytes in varying abundance, as described by Gorer and Boyse (1959a), following injection of parental cells into F1 hybrids (Figs. 5 and 6).

The lymph nodes are smaller than normal and in the later stages of the disease are barely identifiable on gross examination. These tiny structures are firm in consistency and may show considerable deposition of amorphous hyaline-like material and a varying degree of infiltration by histiocytes (Fig. 7). Where these destructive changes are less marked the lymphoid cells show no apparent organization, merely lying in diffuse sheets. Occasionally infiltrations of leucocytes, predominantly lymphoid cells but also polymorphonuclear cells, are seen in the subcapsular zone of the kidney. No cellular infiltrations have been found in the intestinal wall, lung, muscle, skin or brain. The bone marrow and thymus have not been systematically examined but the impression from a few bone marrow specimens is that the granulocytic series of cells predominates abnormally.

(2) Dosage and route of injection of spleen cells. Whereas the intravenous administration of 5 million or more DBA/1 spleen cells to C57BL/6 newborns will cause the prompt death of about 96 per cent of the recipients, reducing the dose below this level will still regularly result in a high percentage of runts although the animals appear to grow to a slightly greater size and to succumb, on the average, a few days later (Fig. 8). Our present experience, considerably larger than at the time of the previous preliminary report (1960), indicates that at doses smaller than 500,000 cells the incidence of runt disease declines sharply but that smaller doses may occasionally produce all the changes typical of runt disease. The smallest number of spleen cells capable of producing the disease has not been accurately fixed and may, of course, be a somewhat variable figure depending upon the relative cellular constitution of the donor spleen at the time the animal is
Fig. 5. Typical microscopic appearance of spleen of animal with advanced runt disease. The normal splenic architecture is greatly distorted and there is a considerable influx of histiocytes. (Haematoxylin and eosin. × 80.)

Fig. 6. High-power view of population of cells in the spleen from an animal with advanced runt disease selected to show the typical histiocyte infiltrate. (Haematoxylin and eosin. × 400.)
Fig. 7. Lymph node from runt sacrificed at one week of age following an intravenous dose of 12 million homologous spleen cells at birth. Extensive cellular destruction is apparent, with considerable amorphous material amongst the surviving cells. The changes in this example are more marked than usual at this age. (Haematoxylin and eosin. × 150.)
sacrificed. There is no evidence that very small doses allow the establishment of tolerance without the damaging effects of a graft-against-host reaction.

Doses in excess of 5 million cells have little influence in hastening the onset of the disease or of death, suggesting a dosage threshold.

In all later experiments doses well above this threshold were accordingly used for consistency.

In contrast to the reliability of the intravenous route the subcutaneous and intraperitoneal routes are much less dependable, as Billingham and Brent (1959) found. Of twelve animals injected intraperitoneally with about 15 million foreign spleen cells only a single one died. This animal did, however, have all
the signs of runt disease. Two may have shown some transient and late effects of the injection but all of the survivors reacted in a normal fashion to grafts of DBA skin when they had reached six weeks of age. Subcutaneous inoculation gave similar results.

(3) Spleen cells from donors previously sensitized to the recipient strain. It is readily possible in the present strain combination to demonstrate acceleration of the graft-versus-host reaction by the use of spleens from donor animals which have previously rejected skin grafts of recipient strain origin (Billingham, 1958). For example, all six of a litter of C57BL/6 animals treated with 15 million DBA/1 spleen cells from donors which had fully rejected bilateral C57BL/6 skin grafts a week previously were dead by the twelfth day. This contrasts with an expected median survival of about 14 days when cells from untreated donors are used. The weight-gain curve shows little difference in these two situations, both of which involve disease of such an acute type that differences are difficult to distinguish.

II. Effect of some specific treatments directed against the foreign cell population

As outlined above, the specific treatments designed to destroy or inactivate only the runt-producing population of spleen cells in neonatal animals with impending runt disease involved either the adoptive transfer of a second inoculum of adult spleen cells from donors isologous with the test animals, or the transfer of serum from isologous adults previously sensitized to DBA/1 tissues. Cells from sensitized donors were also used.

(1) Adoptive transfer of protective cells. Initially the efficacy of adult isologous cells was tested by intravenously administering, on the first day of life, a mixture of approximately equal numbers of DBA/1 and C57BL/6 spleen cells. Almost all animals receiving such a cell mixture, including a dose of DBA/1 cells entirely adequate to produce runt disease under ordinary circumstances, gave no evidence whatsoever of the disease. This was true when
cells were used from either normal adult C\textsubscript{57}BL/6 animals or C\textsubscript{57}BL/6 adults previously sensitized by the skin grafting method described above. Fig. 9 shows the weight-gain record of a representative litter of 6 mice, three of the members of which were treated with 27 million spleen cells in a single injection, half from an adult C\textsubscript{57}BL/6 which had previously rejected bilateral DBA/1 skin grafts and half from a normal DBA/1 donor. Two of the three animals receiving the cell mixture were completely protected from runt disease while the third showed only partial protection. Three controls receiving only the DBA/1 portion of the injection promptly succumbed, as expected, to runt disease. This protective effect, previously observed by Billingham and Brent (1959) and by Siskind and Thomas (1959), is equally strong when the isologous

Fig. 9. Weight-gain record of a litter of 6 newborn C\textsubscript{57}BL/6 mice. Three received 27 million spleen cells, half from DBA/1 adult donors and half from C\textsubscript{57}BL/6 adult donors which had previously rejected DBA/1 skin grafts (open circles). The three controls (solid dots) received only the DBA/1 spleen cells. Two of the three animals receiving the cell mixture were fully protected. One was only partially protected from runt disease and died on the twenty-sixth day.
cells are given intravenously immediately after the homologous cell injection by another vein, indicating that their effect does not depend upon intimate contact before administration. No attempt was made to quantitate the dose of cells required for the protective effect, although the system might be expected to lend itself well to this in a manner similar to Winn’s (1960) use of a parallel experimental design where sensitized cells are mixed with tumour cells before transfer. Later skin grafts applied to the survivors were rejected in an entirely normal fashion with no suggestion either of specific tolerance or of sensitization.

Delaying the protective injection until two days later, or the third day of life, reveals a difference between the rapidity of effectiveness of normal as compared to presensitized cells. Fig. 10 combines the results obtained in the treatment of three litters of C57BL/6 animals all of which received more than 10 million DBA/1 spleen cells intravenously on the day of birth. Two days later approximately 15 million adult C57BL/6 spleen cells were given, again intravenously, 4 animals receiving cells from donors which had previously rejected DBA/1 skin grafts and 5 from normal donors. Although a virtually normal weight-gain curve follows the adoptive transfer of sensitized cells the delay in onset of normal development after the protective injection of normal cells suggests a transient influence of the primary inoculum which is only overcome when the second wave of adult cells reaches an appropriate level of activity. This difference in rate of effectiveness is quite reminiscent of that noted by Billingham, Brent and Medawar (1956) in the time required for rejection of previously tolerated skin grafts by mice receiving normal as against previously sensitized isologous lymphoid cells.

When the second intravenous injection is postponed two days longer to the fifth day of life, unsensitized cells have in no instance been sufficient to overcome the damaging effects of the initial injection. Even the use of cells from donors highly sensitized, as described above, will not release mice under these conditions from
the impending effects of runt disease in every case. Nevertheless, of 28 animals treated in this fashion 19 developed distinctly better than untreated controls, with those that died doing so at an average age of 35 days. Nine survived for over a hundred days and nine died promptly as though unaffected by the second cell injection. DBA/1 skin grafts applied to the long-term survivors were rejected normally. An example of a litter in which a second
injection of sensitized cells at 5 days of life was particularly beneficial appears in Fig. 11. The variation in effectiveness of this late cell treatment appears to occur largely between litters rather than within a single litter and may be attributable in part to differences in the immunological capacity of the cells used in different experiments. Microscopic examination of the spleens and lymph nodes of surviving animals treated in this way at about 4 weeks of age reveals considerable hyperplasia with many active germinal centres.

Fig. 11. Individual weight-gain records of a litter of 6 newborn C57BL/6 mice all of which received an intravenous injection of 12 million DBA/1 spleen cells at birth. On the fifth day of life four animals (solid dots) received a second intravenous injection of 18 million spleen cells from adult C57BL/6 mice which had previously rejected skin grafts from DBA/1 donors. This is a particularly favourable example of the protective effect which can be achieved by a relatively late injection of sensitized cells.
in the lymph nodes and large, prominent Malpighian corpuscles in the spleens.

Further delay of the secondary injection has not been extensively studied. The technical difficulties of performing a satisfactory intravenous injection become rapidly greater as pigmentation deepens in this strain. Intraperitoneal injections of recipient strain cells after the beginning of the "plateau period" have not been beneficial.

(2) Treatment with antiserum directed against DBA/1 spleen cells. There is much evidence that cells of lymphoid origin are particularly susceptible to humoral cytotoxic antibodies in vivo (see, for example, Garver and Cole, 1961).

Although we were initially unsuccessful in our attempts to confirm the finding of Siskind and Thomas (1959) that serum injections from adult members of the recipient strain previously sensitized to lymphoid cells of the donor strain could obviate the onset of runt disease, this observation has since been reproduced repeatedly. Our experiments usually consisted of giving a series of 2 or 3 injections of 0.05 to 0.08 ml. of serum intraperitoneally or intravenously to animals with impending runt disease beginning on the second to fifth day of life.

As portrayed in Fig. 12, a clear difference between the effectiveness of serum from animals sensitized by means of DBA/1 skin grafts and those sensitized with two injections of spleen cells in an adjuvant mixture, as described above, is apparent. Cytotoxic antiserum from animals sensitized by either of the two methods used appeared to be capable of destroying the widely disseminated donor lymphoid cells. A higher titre was apparently achieved by the use of spleen cells and adjuvant, however, than by skin grafting.

Serum derived from spleen cell-adjuvant sensitized donors was roughly as effective as sensitized cells when administered on the fifth day of life. Of a total of 14 animals from four litters treated with serum from spleen cell-adjuvant sensitized donors all 8
members of two litters survived without evidence of disease while the 6 members of the other two litters promptly died of runt disease as though the serum treatment had not been given. Although showing sharp variation in efficacy between litters, it is

![Graph](image)

**Fig. 12.** Mean weight-gain record of 3 separate litters of C57BL/6 mice receiving 10 to 15 million DBA/1 spleen cells at birth. On the second, third and fourth days of life each animal received intraperitoneal injections of 0.05 to 0.07 ml. of serum from C57BL/6 adults previously sensitized to DBA/1 tissues. The upper two curves represent litters treated with serum from animals sensitized by injections of spleen cells in adjuvant into the foot pads. For the lower curve the animals were treated with serum from donors which had rejected DBA/1 skin grafts (see Methods section). The bars represent a single standard deviation of the mean. The "cell-adjuvant sensitized" serum appears to be somewhat more effective in overcoming runt disease.

clear from the data depicted in Fig. 13 that serum treatment can retrieve animals at this stage from lethal runt disease which rapidly overtakes controls in the same litter. Survivors of this treatment reacted normally against DBA/1 skin grafts in every instance when they had reached a suitable size for grafting.
Fig. 13. Weight-gain record of a litter of 7 newborn C57BL/6 mice, all of which received 20 million DBA/1 spleen cells intravenously on the first day of life. Five of these animals were treated on the fifth, sixth and seventh days of life with 0.05 to 0.08 ml. of serum from adult C57BL/6 mice previously injected with a DBA/1 spleen-cell-in-adjuvant mixture. This favourable example shows that such serum injections may be effective as late as the fifth day of life.

III. Effect of some non-specific treatments

This section deals with several forms of treatment, each of which is well known to depress the activity of a variety of cells or, indeed, to destroy them. Although lymphoid cells are particularly sensitive to any of these treatments the rapidly growing newborn animal is generally much more vulnerable to such cytotoxic agents and the revelation of differential effects between the animal as a vehicle and its contained foreign cells may require a fine regulation of dosage.

(i) Cortisone. The retarding effect of cortisone on the homograft reaction is known to vary considerably in strength between species, being relatively weak in the mouse (Medawar and Sparrow, 1956). A dose of 0.025 mg./g. of cortisone acetate was
chosen. This is a large dose, the daily subcutaneous administration of which from birth was alone observed to cause the death of 4 of 10 newborn C57BL/6 mice within the first two weeks. The development of those animals which survived cortisone treatment was only slightly delayed, however. In Fig. 14 the weight gain of 16 runts treated daily with this dose of cortisone is compared with that of 15 animals which had also received 11 to 16 million homologous spleen cells on the first day of life but had not been treated with cortisone. As time goes on a difference between these groups appears which increases until both treated and untreated animals are overcome by the advancing disease. The greatest difference in the mean weights of the two groups was at 16 days but even at 12 days, when a larger number of animals was surviving, a simple t test revealed that the two groups differed.

Fig. 14. Weight-gain curves of two groups of C57BL/6 mice both receiving 11 to 16 million DBA/1 spleen cells intravenously at birth. Each curve expresses the mean weight of the survivors present at each stage and the bars show a single standard deviation of the mean. The numbers in parentheses are the survivors present. The group represented by open circles received 0.025 mg./g. of cortisone acetate subcutaneously each day. A t test of the two groups on the twelfth day indicates a statistical difference between them (P<0.005)
very significantly \((P<0.005)\). Cortisone thus has a mild and transient ameliorating effect on runt disease in these mice.

(2) Whole-body X-irradiation. Whole-body irradiation was also tested for its influence on the course of acute runt disease in these neonatal animals before they had reached the "plateau period". Following the standard injection of more than 5 million DBA/1 adult spleen cells intravenously on the day of birth, a total of 20 C57BL/6 mice were treated at various times with doses of whole-body X-irradiation as follows: 3 received 100 r. on the second day of life; groups of 3 received 50, 100 and 150 r. on the fourth day of life; groups of 4 received 100 and 300 r. on the sixth day of life. (Radiation delivered at 116 r./min. from 50 cm., 250 kv, 15 ma, 1.5 mm. Cu H.V.L.). In every instance these animals died before non-irradiated animals of the same litter also injected with homologous cells. Thus, nothing but a slightly deleterious effect of this treatment is demonstrated with the doses of cells and irradiation chosen.

(3) Burroughs-Wellcome 57-322* (6-(1-methyl-4-nitro-5-imidazolyl)thiopurine). A wide variety of cytotoxic substances have been known for some time to influence the production of antibodies.

This imidazolyl thiopurine derivative was chosen for testing since it has recently been shown to have a relatively low toxicity at doses which appreciably inhibit the inductive phase of agglutinin formation against sheep erythrocytes in mice (Nathan et al., 1961). This finding reinforced similar observations made earlier by Schwartz, Stack and Dameshek (1958) and by Sterzl (1960, 1961) that 6-mercaptopurine, and some related compounds, will greatly suppress the inductive phase of antibody production.

Continuing administration of B.W. 57-322 and 6-mercaptopurine has also recently been found to prolong the survival of renal homografts in dogs (Calne and Murray, 1961) although the

* Generously supplied by Dr. George H. Hitchings of Burroughs Wellcome Co. (U.S.A.) Inc.
doses required for best results involve a high incidence of toxic reactions in the treated recipients.

Preliminary observations of a few normal C\textsubscript{57}BL/6 mice given subcutaneous injections of B.W. 57–322 on the first, third and fifth days of life indicated that almost all animals receiving as much as 25 mg./kg. on each of these days were dead by the tenth day of life.

Toxicity on a similar dosage schedule with 12 mg./kg. or less was considerably milder with all animals surviving after treatment with 3 mg./kg. Five animals were thus treated with three injections of 15 mg./kg. and seven with 3 mg./kg. of this drug after neonatal injections of 19 million DBA/1 spleen cells. No delay in onset of runt disease or amelioration of its severity was observed as compared with control animals of the same litters. All were dead by the twelfth day. A similar series of injections of 1.5 mg./kg. also failed to influence the disease.

(4) Amethopterin (methotrexate; 4-amino-\textsuperscript{10}-methylpteroylglutamic acid). This antifolic acid compound has proven to be of use in the treatment of certain leukaemias and other malignant diseases. Its effect on the inductive phase of serological antibody production has been reported to be slight (Nathan \textit{et al.}, 1961) although aminopterin, another folic acid antagonist, has been found to suppress antibody production appreciably (Sterzl, 1961).

Uphoff (1958) has found this compound to be capable of procuring a considerable increase in the percentage of mice surviving the delayed reaction following lethal whole-body irradiation and subsequent infusion of adult homologous bone marrow. She used a series of nine intraperitoneal or subcutaneous injections giving doses of 3.0 mg./kg. or 1.5 mg./kg. at each injection. Fairly severe drug toxicity occurred with these treatments and was more marked in females, as previously observed by others (Goldin \textit{et al.}, 1950).

Very recently she has extended these observations by demonstrating that a similar course of amethopterin treatment, combined
with a series of three homologous spleen and thymus cell injections over a one-week period, will render mice specifically more susceptible to transplantable tumours of donor origin transferred two or more weeks following treatment (Uphoff, 1961). This phenomenon has been interpreted as a form of true "immunological tolerance". There are also other recent reports of the depressant effect on the homograft reaction of continued administration of this compound (see Blumenstock et al., 1961; and Humphreys et al., 1961).

Our results with this drug have been striking. Initial toxicity tests were done, again with three equal doses given subcutaneously to newborn C57BL/6 mice on the first, third and fifth days of life. Three doses as small as 0.75 mg./kg. resulted in a one hundred per cent mortality.

With half of this dose (0.37 mg./kg.) 2 of a test group of 14 animals died, one on the fourteenth and one on the twenty-sixth day. The remaining animals developed somewhat more slowly for about the first month, but they promptly recovered with no obvious residual effects except for a distinct greying of the hair, particularly noticeable over the ventral surface. Eight of these animals were grafted with DBA/1 skin at about six weeks of age. All of these grafts were fully rejected by the eleventh day.

Table I

<table>
<thead>
<tr>
<th></th>
<th>No. animals</th>
<th>Deaths</th>
<th>Deaths</th>
<th>Survivors</th>
<th>Survivors</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>after 25 days</td>
<td>tolerant</td>
<td>highly tolerant</td>
</tr>
<tr>
<td>Runts (no additional treatment)</td>
<td>140</td>
<td>96</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Potential runts (treated with amethopterin 0.37 mg./kg. x 3)</td>
<td>41</td>
<td>41</td>
<td>45</td>
<td>89</td>
<td>54</td>
</tr>
</tbody>
</table>

All untreated runts received in excess of 5 million DBA/1 spleen cells neonatally. Potential runts treated with amethopterin received more than 15 million DBA/1 spleen cells. The survivors were later grafted with DBA/1 skin. Skin graft survival of more than 15 days was considered to indicate some degree of tolerance. Survival of more than 30 days was designated a "high degree" of tolerance.
A series of 41 newborn C57BL/6 animals was then treated with three injections of 0.37 mg./kg. (or a total of about 2 μg. per

17,400,000 DBA CELLS

G.

0.37 mg/kg methotrexate s/c days 1, 3 & 5

Untreated Control

0.75 mg/kg methotrexate s/c day 1

Days of Age

Fig. 15. Individual weight-gain records of a litter of 7 newborn C57BL/6 mice all of which received 17 million DBA/i spleen cells on the first day of life. The control died promptly of runt disease. Single injections of amethopterin (methotrexate) (0.75 mg./kg.) were ineffective in checking the onset of runt disease. Three injections of amethopterin (0.37 mg./kg.) given on the first, third and fifth days of life allowed four animals to develop at a normal rate.

mouse) over the first five days of life following neonatal injections of approximately 15 million DBA/i spleen cells. As indicated in Table I, 59 per cent of these animals survived. Those which died tended to do so later than untreated runts. Although many
Fig. 16. Four survivors of the litter represented in Fig. 15 (T-204). All accepted DBA/1 skin grafts at about 6 weeks of age. The grafts have remained in excellent condition for 95 days indicating a high degree of tolerance.
of the ultimate survivors showed slight temporary signs of disability, most grew at very nearly a normal rate to become quite normal in outward appearance except for the loss of pigmentation previously mentioned (Fig. 15). A few trials of single doses of 0.75 mg. on the day of birth shortly after cell injection proved ineffective. When grafted at six to seven weeks of age, 54 per cent of these surviving animals were found to be highly tolerant of DBA/1 skin grafts (i.e. survival for more than 30 days) some of which have been observed as long as 95 days (Fig. 16). Three of these tolerant animals were also grafted with skin from A-strain donors and in each case these grafts were fully rejected by the eleventh day. Four other animals, bearing long-term homografts of DBA/1 skin, were killed and their spleens and lymph nodes transferred as cell suspensions to individual normal adult C57BL/6 mice by intraperitoneal injection. DBA/1 skin grafts applied five days thereafter as a "chimera test" according to the design of Mitchison (1956) were fully destroyed in seven to eight days confirming that the original tolerance-conferring inoculum, or at least some of its descendant cells, had survived. Doses smaller than 0.037 mg./kg. have not so far been used.

IV. Secondary transfer of cells from runts

Our preliminary efforts to transfer runt disease by injecting spleen cell suspensions from animals with the disease into newborn recipients of the same strain can be summarized as follows. A total of 18 recipients have so far received injections of pooled spleen cells from runts in their sixth or seventh days of life. Each received from 12 to 32 million nucleated cells intravenously. In no case has any distinct retardation of development in the injected animals been observed.

Injection of a portion of the cell suspensions used in these experiments into adult C57BL/6 recipients results in subsequent accelerated rejection of DBA/1 skin grafts, thus demonstrating the presence of DBA/1 antigens. When they have reached an
adequate size DBA/1 skin grafts have also been applied to the secondary recipients. No significant deviation from a normal rejection was observed in any case. Some of the animals receiving the larger cell doses have not yet been skin grafted. It would appear likely that failure to demonstrate tolerance in these animals is the result of an insufficient initial dosage of DBA/1 antigens. The failure to demonstrate runt disease might be from the same shortcoming or, at least partially, because of a reduction in the capacity of the foreign cells to react against recipient-strain antigens. Further work is now in progress to clarify this question and some discussion of this point appears below.

Discussion

As one looks at the overall course of the graft-versus-host reaction in this experimental arrangement the first clear point is that the reaction is not an immediate one. Much as in the typical host-versus-graft reaction there is a period of several days before disease is manifest. Thereafter it is generally agreed (de Vries and Vos, 1959; Gorer and Boyse, 1959a) that the reaction rapidly embarks on a complex chain of pathological events. Widespread derangement of the cellular machinery of response to foreign substances may well allow the entry of pathogenic organisms in some cases. An outstanding cause of debility in the animals of the present study must have been hepatic damage. Our observations here reaffirmed the possible importance of the large macrophage or histiocyte as a possible operative cell in the production of the disease (see Gorer and Boyse, 1959a).

The facts that specific treatments directed toward destroying the cells bearing donor-strain antigens can retrieve some animals from an otherwise inevitable death when begun as late as the fifth day of life, and that animals which survive this treatment are not tolerant of donor-strain skin, indicate that an appreciable share of the irreversible damaging effects of these cells takes place after the
fifth day. This has not been interpreted as evidence against the "exhaustive sensitization" concept advanced by Simonsen (1960) who has presented evidence that adult spleen cells transferred to F₁ hybrids between the donor and an unrelated strain are rapidly and specifically rendered incapable of reacting against their host. This view has since been supported by the findings of Dineen (1961) who was largely unable to transfer runt disease to secondary recipients even though they were found to contain donor antigens. If we enlarge our considerations to include radiation chimeras, findings of similar import by Cole and Davis (1961) can be included. These authors demonstrated that cells of donor origin in long-lived radiation chimeras can be specifically incapable of reacting against host antigens. The protracted course of runt disease in some strain combinations and the long delayed onset of secondary disease after irradiation and homologous bone marrow transfer, taken with the present findings, make it unlikely that a high proportion of the donor's ability to react to the host is very promptly destroyed or inactivated. From the evidence available it would appear, however, that graft-versus-host reactivity often gradually disappears.

Indeed, this phenomenon may help to explain the response of, for example, a "conventionally tolerant" mouse to skin grafts from certain third strains. As Billingham and Brent (1959) found, and we have confirmed, A-line mice made tolerant of CBA skin grafts by the neonatal injection technique will reject C₃H skin grafts, but not in the usual 10 to 11 days characteristic for C₃H grafts on normal A-line recipients nor the 12- to 13-day period expected of C₃H grafts on normal CBA mice. Instead such C₃H grafts are rejected after 20 to 25 days. Sharing of antigens between C₃H and CBA might account for a relative reduction in reactivity of the indigenous or A-line components of the chimeric animal's lymphoid system, and some reduction in cell number consequent to a mild graft-versus-host reaction may account for an additional amount of depression. The CBA
portion of the chimera should not, however, be so inactivated unless it had become tolerant of its A-line surroundings which contain several of the antigens that are present in C3H but missing in CBA. The prolonged survival of these C3H grafts may, therefore, be at least partly explained by a mechanism of graft-versus-host tolerance.

It must remain entirely speculative as to whether this change reflects the specific depletion of reactive cells by allergic destruction as considered by Gorer and Boyse (1959b), or by some other of the alternatives which have been proposed to account theoretically for immunological tolerance (Medawar, 1960).

The fact that the cytotoxic agent amethopterin appears greatly to hasten this process in some animals is unfortunately not critically helpful in clarifying its mechanism. Although a number of cell types are doubtless injured by this drug (certainly pigment cells) blind destruction of an unselected segment of the lymphoid cell population of the young animal may be expected to do no more than change the relative proportion of graft and host cells. No indication was forthcoming, however, from our cell dosage experiments that very large or very small single doses had any influence on the establishment of a foreign cell population tolerant of the injected host. One must speculate, therefore, that a more particular action of this drug exists and that the function of cells active in homograft reactions of this sort is particularly susceptible to its effects in an unknown way. This additional damaging influence, affecting the appropriate cells in the presence of antigen excess, must persist long enough either to dispose of a sufficient proportion of the offending cells or to redirect their course of action so that they become innocuous to the host. This would result in a selective elimination, by one way or another, of immunologically active cells but would still require that tolerance be maintained in the constantly self-replicating cell population, presumably in large measure by virtue of the presence of large quantities of the appropriate antigens. It is a matter of considerable
interest that something of this kind actually does occur since the graft-against-host reaction is not merely delayed but is held in permanent abeyance.

Whatever may be the mechanism of this intriguing phenomenon it now seems apparent that “tolerance” can be induced in a population of lymphoid cells from an adult donor to histocompatibility antigens of animals of considerable genetic diversity and that this process can be promoted by amethopterin treatment.

Acknowledgements

This work was supported by grants A-4497 and GSF-5110-C1-B from the United States Public Health Service. I should like to express special appreciation to Miss Mary L. Wood for her expert technical assistance. Thanks are also due to Mr. Eric Grave for assistance with the photographs and to Miss Margaret Wilkie for help with the manuscript.

REFERENCES

**DISCUSSION**

Simonsen: I am very convinced by Dr. Russell’s evidence that nothing like complete tolerance of the graft is induced instantaneously in his system, that the graft in fact goes on reacting against the host for some days. The graft-versus-host tolerance which I described occurring in 24 hours in the C3H and C3H × ST/A system, I wouldn’t claim was 100 per cent tolerance but it was a very marked partial tolerance; my best estimate is that about five-sixths of the reactivity towards ST/A antigen got lost in the first day. But I think the question of whether the animal is going to die or not may be a very marginal question in terms of cells which continue reacting after the first day. It may very well be that a relatively small fraction of the original graft is decisive. And it may be this fraction which Dr. Russell picks up with his treatment of antiserum or with sensitized cells. If this is true, his test system may in fact be more
sensitive than the spleen assay system to detect the residual reactivity of the grafted cells against the host.

Medawar: I think it would be valuable in this connexion to ventilate a point which I have discussed privately with Dr. Simonsen, namely the technical validity of the serial passage of spleen cells in studying the propagation of runt disease or splenomegaly. Suppose one injects adult lymphoid cells into newborn animals to cause splenomegaly and runting: if the lymphoid cells which actually react against the host undergo the kind of transformation that Dr. Gowans has described, they may turn into cells which are perhaps stickier and less easy to get out of the spleen when one wants to make a cell suspension, or into cells which, with a lot of cytoplasm, are more vulnerable to handling. So that I don’t think one can tease or squeeze the cells out of enlarged spleens and assume they are necessarily a fair sample of the reactive population. This seems to me to be, perhaps an illusory difficulty, but anyhow a difficulty of principle in using serial passage of cells to estimate the immunological potencies of cells taken from enlarged spleen.

Simonsen: I don’t think there is much difficulty in getting the cells out of the spleen. Perhaps more would disintegrate, in this process, in enlarged spleens than in normal spleens, but I think your objection would not apply to the spleen removed at one day after injection which is not yet an enlarged spleen at all.

Medawar: These transformations described by Gowans occur with incredible rapidity. Still, we don’t really know enough about this to discuss it. I’m merely raising it as a theoretical point.

Billingham: I was interested to hear of Dr. Russell’s difficulty in transmitting runt disease from one runt to another potential host. We’ve tried to “passage” this disease in rats and have had only sporadic successes. Buffy-coat leucocytes pooled with suspensions of spleen and node cells were transferred from runts. Sometimes the disease appeared consistently in every member of the host litter but we never managed to get beyond the first passage.

Dr. Russell, have you compared the runt-causing effects of lymph node cells with those of spleen in your very sensitive system?

Russell: We have done just a little work along these lines. We believe that lymph node cells are more effective than spleen cells in equal numbers.
**Billingham:** Dr. Silvers and I have done this with the CBA to A mouse strain combination where splenic cells at the 7 million cell dosage level only cause about 45 per cent mortality through runt disease, whereas 1 million axillary or brachial node cells cause about 70 per cent mortality, and almost 100 per cent of the recipients of 2 million node cells succumb. We wondered why increasingly high dosages of spleen cells failed to increase the incidence of runt disease above 50 per cent in view of the number of immunologically competent cells that must be present. We therefore tried to simulate a spleen cell suspension by mixing a potentially lethal dosage of 2 million node cells with about 10 million bone marrow cells. The presence of the latter significantly lowered the mortality that would have been caused by the node cells alone, through a sort of “buffering” action. When attempting to transfer runt disease, one is inevitably transferring “activated” immunologically competent cells with a considerable number of non-immunologically competent cells that may be of donor or host origin. These may exert a similar sort of buffering effect, so that overt runt disease fails to appear. This postulated buffering action may be the outcome of a competition for sites in their new host on the part of cells of different lineages.

**Voisin:** Cytologists tell us that the spleen in the rodents is cytologically like a mixture of bone marrow and lymph nodes and this might explain some of your results.

**Michie:** About the buffering effect—you were also varying the total cell dosage, weren’t you, so that it might have been an overflow effect, dependent on your total cell dose?

**Billingham:** That is possible.

**Brent:** But what happens to the cells when they “overflow”—where do they go?

Medawar, Brown and I have attempted to eliminate the donor’s cells from tolerant adult mice—mice which had been made tolerant by neonatal injection. In adult mice the transfer of large volumes of hyperimmune serum doesn’t appear to have any adverse effect on the persistence of the donor’s cells. There seems to be a real difference here which may depend on either the age of the animal or the age of the donor cell, presumably the age of the animal. When you continue your attempts to remove donor cells in older mice it will be very interesting to see when you get a fadeout, if you get a fadeout at all.
Russell: As far as the effect of serum is concerned, these differences are entirely a mystery to me. I didn’t expect it to be as effective and, as I said, we didn’t get success with it in early experiments. I plan to go on and try the effect of antiserum in abolishing tolerance in C57 mice induced by F1 hybrid cells between DBA and C57. It might also be worth while to see if we can abolish the tolerance we achieve to homozygous DBA/i tissues using amethopterin.

Medawar: Your doses of serum are enormous, aren’t they?—about 5 per cent of the body weight.

Russell: They are.

Brent: I wonder whether amethopterin would have an effect on runting produced by presensitized cells. Perhaps the cells which are undergoing the process of sensitization are particularly susceptible to the drug and are knocked out as they are being sensitized. If this is true you would abolish the runting effect and leave in the host only cells which had not been stimulated by the antigens. There must be a lot of cells in lymphoid tissue which do not react immunologically, and these would continue the tolerance-inducing stimulus; this explains the fact that quite a few of your survivors were tolerant though they didn’t show the runting syndrome.

Russell: Your thinking is very similar to my own, and this suggestion is certainly very worth while; I haven’t done such an experiment as yet. Another one along similar lines would be to treat the donor animal with the drug before using it as a donor.

Voisin: Do you know anything about the selective effects of either the drug or irradiation on the immunologically competent cell population? What are the cells which are the most readily destroyed or impaired and is there any known order of action?

Russell: I cannot answer this. I would be very interested to know an answer.

Mitchison: I don’t see the discrepancy between Dr. Brent’s and Dr. Russell’s results with antiserum. May not the antibody, so to speak, shoot down the missiles after they have already left the launching site? If Dr. Russell is agglutinating lymphocytes in the circulation, this might procure a protective effect long after tolerance has been achieved.

Brent: Presumably you have tested your animals for the presence of donor cells after treatment with antiserum?
**Discussion**

**Russell:** Yes, but only by skin graft tests for tolerance, which may have missed some persisting cells.

**Brent:** It would be interesting to know whether or not you are actually eliminating the donor cells or whether you are simply reducing the cell population to a permissible level.

**Russell:** Exactly. I think that is something we must try to find out about.

**Hašek:** Have you tried to abolish tolerance by using serum antibodies that are concentrated—for example, γ-globulin concentrated on DEAE cellulose or by some other concentration technique?

**Russell:** I haven’t tried this yet.

**Hašek:** We can abolish tolerance in heterologous systems using large doses of serum. These sera destroy cellular chimerism in tolerant animals and lead to the destruction of tolerated skin grafts. Both the cytotoxic components and heteroprecipitins take part in the reaction; the latter are responsible for the early vascular-necrotic changes in the graft. If we use γ-globulin instead of the large quantity of serum, it also works well (Hašek, M. [1962]. *Folia biol. (Prague)*, 8, 57). Thus I feel that it would be worth while trying once again the effect of serum antibodies in the homologous systems using concentrated antibodies.

**Hildemann:** If each competent lymphoid cell injected is capable of producing a certain quantum of damage in the neonatal host, it would seem surprising that normal lymphoid cells at varying doses should not give different median survival times. If I understand Dr. Russell correctly, he obtained the same median survival times as we did with small lymphocytes over a comparatively wide dosage range. However, when preimmunized lymphocytes are injected then accelerated death follows. Is this perhaps because the normal lymphocytes carry a semi-automatic rifle and the immune ones a machine gun? Or does this relate to the number of competent replicating cells present and therefore the intensification of the attack? The dosage relationships are not as clear-cut as one might expect. As I recall the results of Billingham and colleagues with rats, though they gave no median survival times, the time distribution of runt disease in the rat appears to be very similar to that in mice, that is median survival times around 16–17 days. So it would appear that the time course of events leading to death is much the same
DISCUSSION

whenever a certain threshold dose of lymphoid cells is exceeded in the original inoculum.

Billingham: That is certainly true of rats injected with thoracic duct cells or lymph node cells. Above a certain threshold dose the clinical events are remarkably constant.

Nakić: I would like to make a point about the inactivation of donor cells, or as some might call it, “allergic death” of donor cells.

As Dr. Russell pointed out, there is something strange about it. One lethally irradiates an animal and injects it with homologous bone marrow. For a time the functional presence of donor cells is evident only in their beneficial effect on the survival of the irradiated host. But several months later, these same donor cells “turn wicked” and cause fatal secondary disease. One would think that they have just been gathering momentum for an all-out offensive against the host.

We have had a similar experience in our experimental model. Consider a separated parabiont dying of “parabiotic disease”. Here donor cells, despite the fact that they are in contact with an immense quantity of antigen, not only do not succumb to “allergic death”, but are quite capable of killing the host. One would expect that in such a situation, the whole of the donor cell population would be engaged in the offensive, but it is not so; if such a sick parabiont had also been challenged with a skin graft from the third strain, donor cells would have a sufficient reserve force to deal with this graft too.

I would also like to add a few words regarding the relationship of runting to tolerance: I believe that runts are tolerant animals—at least no one has ever shown that they are not. Chronic runts are tolerant animals, and all animals dying of “secondary disease” and “parabiotic disease” are tolerant animals. Going back to Dr. Hildemann’s paper, I would think that, despite his contention to the contrary, he did induce tolerance by the use of small lymphoid cells simply by inducing fatal runting.
RÔLE OF THE THYMUS IN TRANSPLANTATION TOLERANCE AND IMMUNITY

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Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London

It has often been debated whether the thymus really plays any significant part in immunological processes. The intact thymus has not been shown to produce antibody in immunized animals nor does it undergo the histological changes observed in lymph nodes and spleen following the parenteral administration of antigens (Fagraeus, 1948; Askonas and White, 1956). Furthermore, thymectomy in the adult animal has been associated with slight or no significant depression of antibody production. On the other hand, there are hints from experimental and clinical observations that the thymus may play some rôle in the control of the immune response. Thus, it is well known that the thymus is the major lymphoid organ in perinatal life at a time when the animal is most sensitive to external modification of the immunological system. In acute infections, when presumably the need for antibody production is great, the thymus undergoes rapid involution and in patients with acquired agammaglobulinaemia there is often the simultaneous occurrence of benign thymomas.

Our interest in the thymus arose from observations made independently in this laboratory (Miller, 1959) and in two other laboratories (Gross, 1959; Levinthal, Buffett and Furth, 1959) that thymectomy in mice as late as one month of age would prevent viral induction of lymphocytic leukaemia without actually removing the virus that had been introduced at birth. This prompted us to do certain experiments which required the
use of thymectomized newborn mice (Miller, 1962). It then became evident that the thymus at an early stage in life plays a very important part in the development of immunological response. A preliminary communication of this work has been published (Miller, 1961).

Materials and methods

Mice

Mice of the following inbred strains were used: Ak (H-2\(^k\)), C\(_3\)H (H-2\(^k\)), C\(_5\)7BL (H-2\(^b\)), BALB/c (H-2\(^d\)), DBA/2 (H-2\(^d\)), T6 (?H-2\(^k\)), and (Ak × T6) F\(_1\) hybrids.

Thymectomy

The mice were thymectomized within a few hours of birth unless otherwise indicated. The operation was performed under light ether anaesthesia. Part of the sternum was excised and the thymic lobes were sucked out of the thorax by means of a glass pipette connected to a negative pressure system. Three or four interrupted black silk sutures were used to close the skin edges. The immediate operative mortality was no higher than 15 per cent but the mortality from cannibalism or neglect was considerable. Control littermates were sham-thymectomized, that is they underwent the full operative procedure including excision of part of the sternum but the thymic lobes were left intact.

Thymus grafting

Thymuses from newborn donor mice were removed aseptically and introduced by a sterile trocar into the subcutaneous tissues under the right axilla of two- to three-week-old recipients.

Skin grafting

Skin grafts from 1- to 2-month-old mice of either sex or from 2- to 3-week-old Wistar rats of either sex were transplanted to
Adoptive immunization

Normal 2-month-old C3H female mice were immunized against normal Ak tissues, each mouse receiving bilateral skin grafts and an intraperitoneal injection of cells from 2 thymuses and 2 spleens of 1- to 2-month-old healthy Ak donors. Seven to eight days later the mice were killed and cell suspensions were prepared from their axillary and inguinal lymph nodes and spleen. From 20 to 50 million such cells were injected intraperitoneally into appropriate recipient mice.

Blood counts

Differential and absolute white cell counts were performed on tail vein blood at 6 weeks of age.

Histology

Sections were fixed in Bouin’s fluid and in bufferred neutral formalin. They were stained routinely with haematoxylin and eosin and where necessary with Unna-Pappenheim stain.

Results

Weight curves and mortality

The body weights of mice thymectomized and sham-thymectomized at birth were recorded at intervals during the first three weeks of life and are shown in Fig. 1. It can be seen that there is no significant difference between the two groups.

Mortality in the thymectomized group was high after two months of age, as many as fifty per cent of the mice in some strains dying from a syndrome which, clinically, closely resembled the runting syndrome. This was characterized by progressive
Fig. 1. Body weights of BALB/c mice thymectomized (○) and sham-thymectomized (×) at birth.
loss of weight, lethargy, ruffled fur, hunched posture, diarrhoea and death within two to three weeks.

**Peripheral blood**

Absolute and differential leucocyte counts were performed on tail blood at 6 weeks of age in healthy mice that had been thymectomized or sham-thymectomized at birth. The total white cell count in the thymectomized group was half that in the sham-thymectomized controls, the decrease being entirely due to a lymphopenia (Table I). There was no significant difference in the levels of the polymorphonuclear neutrophils, eosinophils and monocytes.

**Histology of lymphoid tissue**

Neonatally thymectomized, healthy-looking, 6-week-old mice were killed and their tissues examined histologically. All these mice showed one striking anatomical abnormality: involution of the lymphoid tissues. The spleen was greatly reduced in size and the average spleen weights were fifty per cent below those of sham-thymectomized control mice of the same age. Histological examination revealed ill-defined, inactive follicles (Figs. 2 and 3) showing little basophilia and few mitoses. The lymph nodes were also considerably diminished in size and showed inactive follicles with poor cellularity (Figs. 4–7). There were no germinal centres

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**Table I**

<table>
<thead>
<tr>
<th></th>
<th>Mice thymectomized at birth</th>
<th>Sham-thymectomized at birth</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leucocytes</td>
<td>5640±320</td>
<td>8730±260</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>per mm.³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2880±200</td>
<td>6600±720</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>per mm.³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1970±210</td>
<td>1390±140</td>
<td>Not significant</td>
</tr>
<tr>
<td>per mm.³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils and</td>
<td>690±60</td>
<td>700±70</td>
<td>Not significant</td>
</tr>
<tr>
<td>monocytes per mm.³</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Fig. 2. Spleen of 6-week-old (Ak × T6)F₁ mouse sham-thymectomized at birth. (× 10.)

Fig. 3. Spleen of 6-week-old (Ak × T6)F₁ mouse thymectomized at birth. (× 12.)

Fig. 4. Inguinal lymph node of 6-week-old (Ak × T6)F₁ mouse sham-thymectomized at birth. (× 16.)

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Fig. 5. Inguinal lymph node of 6-week-old (Ak x T6)F₁ mouse thymectomized at birth. (× 16.)

Fig. 6. Inguinal lymph node of 6-week-old (Ak x T6)F₁ mouse sham-thymectomized at birth. (× 110.)
Fig. 7. Inguinal lymph node of 6-week-old (Ak×T6)F₁ mouse thymectomized at birth. Note poor cellularity. (×110.)
Fig. 8. Three-month-old (Ak×T6)F₁ mouse thymectomized at birth and grafted at 6 weeks with skin from C₃H (upper left), C₅7BL (upper right), BALB/c (lower left) and DBA/2 (lower right).

Fig. 9. Three-month-old (Ak×T6)F₁ mouse thymectomized at birth and grafted at 6 weeks with skin from Wistar rat (upper) and C₃H (lower).
and there was often a deficiency of plasma cells in many of the lymphoid tissues examined.

Autopsies were performed on neonatally thymectomized mice that died as “runts” between 2 and 4 months of age. The involution of lymphoid tissue in these mice was extreme. The lymph nodes were minute and showed no proper structure and no plasma cells. Sometimes they were reduced to a small piece of adipose tissue containing only a few lymphoblasts. The spleens were markedly shrunken and the follicular structure had disappeared. There were few or no lymphocytes and no plasma cells. The Peyer’s patches were poorly developed, inactive and deficient in lymphocytes.

Survival of allogeneic skin grafts

The survival of allogeneic skin grafts in mice thymectomized at birth, at 4 to 7 days, at 3 weeks, in mice sham-thymectomized at birth and in entirely normal mice is shown in Tables II and III. It can be seen that thymectomy in the neonatal period was associated with prolonged survival of skin grafts not only from donors which differed at the H-2 locus (Fig. 8) but also from donors of a different species (Fig. 9). These grafts all grew normal tufts of hair. Rejection of grafts, when it occurred, was characterized by gradual diminution in size of the graft and progressive thinning of the hair until the graft eventually disappeared. There were no signs such as thickening, reddening, oedematous swelling, bleeding or scab formation.

None of the mice that rejected skin grafts by 25 days and only a few of those that rejected grafts of mouse origin by 60 days died as runts. All the mice that carried rat skin for longer than 25 days became runted and died with the graft intact. The majority of the deaths from runting occurred in the group of mice that were tolerant of foreign grafts for more than 60 days.
Table II

SURVIVAL OF ALLOGENEIC SKIN GRAFTS IN THYMECTOMIZED C3H MICE

<table>
<thead>
<tr>
<th>Treatment given</th>
<th>Donor skin</th>
<th>Number of C3H mice grafted</th>
<th>Number of grafted mice showing skin graft survival for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;25 days</td>
</tr>
<tr>
<td>Thymectomized at birth</td>
<td>Ak</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Sham-thymectomized</td>
<td>Ak</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Thymectomized at 4-7 days</td>
<td>Ak</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Thymectomized at 3 weeks</td>
<td>Ak</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>11</td>
<td>61</td>
</tr>
<tr>
<td>Untreated</td>
<td>Ak</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>4</td>
<td>4</td>
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</table>
Table III

**Survival of allogeneic skin grafts in thymectomized (Ak × T6)F<sub>1</sub> mice**

<table>
<thead>
<tr>
<th>Treatment given</th>
<th>Donor skin</th>
<th>Number of (Ak × T6)F&lt;sub&gt;1&lt;/sub&gt; mice grafted</th>
<th>Number of grafted mice showing skin graft survival for</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;25 days</td>
</tr>
<tr>
<td>Thymectomized at birth</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;57&lt;/sub&gt;BL</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
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<td>3</td>
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<tr>
<td></td>
<td>DBA/2</td>
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<tr>
<td></td>
<td>Rat</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Sham-thymectomized</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C&lt;sub&gt;57&lt;/sub&gt;BL</td>
<td>5</td>
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</tr>
<tr>
<td></td>
<td>BALB/c</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Thymectomized at 3 weeks</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H</td>
<td>12</td>
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</tr>
<tr>
<td></td>
<td>BALB/c</td>
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<td>4</td>
</tr>
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</table>
Adoptive immunity

Four neonatally thymectomized C3H mice bearing healthy Ak skin grafts were injected one month after grafting with C3H lymphoid cells from non-thymectomized donors presensitized against Ak tissues. In all four mice a severe reaction was evident in the skin graft 12 days after the injection of sensitized cells.

Thymus grafting

Four neonatally thymectomized (Ak × T6)F1 mice grafted two weeks after birth with an intact day-old Ak thymus were grafted at 8 weeks of age with skins from C3H, C57BL, BALB/c and DBA/2 donors. In all four mice DBA/2 and BALB/c skins were rejected between 15 and 30 days after grafting, C57BL between 30 and 35 days and C3H between 35 and 40 days. These data suggest, but do not prove, that immune reactivity might be restored to neonatally thymectomized mice by thymus grafting.

Discussion

It has been shown that thymectomy of the neonatal mouse is associated with severe depletion in the lymphocyte population and serious immunological defects in the mature animal. One is tempted to suggest that during foetal life, and possibly even in postnatal life, the thymus produces the originators of immunologically competent cells which mature and migrate to other sites (Miller, 1961). The results presented here provide strong support for the predictions made recently by Burnet (1961) and Auerbach (1961) that the thymus may represent the major primordium of the immunological system. They are in harmony with the recent experimental findings of Mueller, Wolfe and Meyer (1960) that neonatally bursectomized chicks could not produce antibodies, and of Archer and Pierce (1961) that rabbits thymectomized at birth subsequently failed to show any antibody response to bovine serum albumin.

One basic feature of the states of immunological failure,
whether classical immunological tolerance, immunological unresponsiveness, or even Felton's immunological paralysis, appears to be the functional absence of antibody-forming cells necessary for reacting with the particular antigens concerned. The physical absence of antibody-forming cells seems to characterize the neonatally thymectomized mouse. It would be satisfying to think that when one is inducing a state of immunological tolerance one is in effect performing a thymectomy, not a total thymectomy, nor a thymectomy in the surgical sense, but a partial, selective, functional thymectomy. In other words, injected cells or antigens might make contact with certain cell types differentiating in the thymus and in some way prevent these cells from maturing to a stage when they would be capable of reacting immunologically.

To gain support for such a theory, it would be necessary to show that injected cells or antigen can indeed find their way to the thymus, that different immunological faculties mature at different times, that immature cells are much more susceptible to functional elimination than mature cells, and that cells actually do migrate out of the thymus to other tissues.

There are some well documented data which suggest that nothing that is injected into a rodent ever finds its way into the thymus. For instance, Gowans, Gesner and McGregor (1961) showed that tritium-labelled small lymphocytes injected into a rat exhibit what is generally known as a homing instinct. That is, they settle in the lymph nodes, in the white pulp of the spleen, in the Peyer's patches and in the bone marrow. They cannot, however, be found in the thymus. Marshall and White (1961) showed that trypan blue or pneumococcal polysaccharide or other antigen when injected parenterally into guinea pigs was not taken up by the normal thymus. These studies suggest the existence of a barrier against the entry of cells or antigenic material into the thymus. Reasoning teleologically, one can appreciate the significance of such a barrier, if it is conceded that immunologically competent cells are differentiating in the thymus even in postnatal
life and are particularly susceptible to functional elimination whilst in their immature state. But how can the existence of such a barrier be reconciled with the suggestion made above that injected antigenic material acts as a tolerance-conferring stimulus by eliminating in some way certain cell types originating in the thymus? Experimental results, however, do not conflict with this theory as the existence of a thymus barrier has only been demonstrated in the adult animal, and experiments by Billingham and Brent (1959) have shown that the thymuses of mice rendered tolerant by neonatal injection of foreign cells all contained a demonstrable population of these cells.

The next point one would like to investigate is whether different immunological faculties are maturing at different times or at different rates. Recent data obtained in our laboratory suggest that the earlier in life a thymectomy is performed the less competent the mouse becomes to reject skin grafts of increasingly greater "foreignness". Furthermore, when the capacity to reject foreign skin grafts is restored to neonatally thymectomized mice, possibly as a result of thymus grafting, skin from donors that are genetically least related to the host is rejected first while skin from donors that are genetically most closely related to the host comes off last.

There is much to suggest, but no real direct evidence to prove, that lymphocytes do actually leave the thymus. For instance, mitotic counts are 4 to 6 times higher in the thymus than in other lymphoid organs and DNA turnover is 2 to 5 times as active in the thymus as it is in lymph nodes (Bierring, 1960). Under normal conditions, cell death in the thymus is slight, amounting to only about 15 per cent of total cell production (Kindred, 1942). Thymectomy in the adult animal is generally associated with some diminution in the lymphocyte population of the thoracic duct lymph, peripheral blood, lymph nodes and spleen (Bierring, 1960; Metcalf, 1960). Thymectomy at a very early age is accompanied by more marked decrease in the lymphocyte content of the
thoracic duct and lymph nodes (Schooley and Kelly, 1958). It could be argued from these results that the lymph nodes and spleen are, therefore, important destinations for thymus lymphocytes. On the other hand, these effects of thymectomy might be related to the production by the thymus of a lymphocytosis-stimulating factor (Metcalf, 1958) which may to some extent control lymphopoiesis in lymphoid organs.

Transfused thymus cells have been traced to the red pulp of the spleen, particularly perifollicularly, at the site where plasma cell proliferation is known to take place during antibody formation (Fichtelius, 1960). This led to the suggestion that the thymus and the spleen may together constitute a large type of central lymph node, the thymus producing lymphocytes and the spleen forming antibodies with their aid. By using appropriate chromosome markers, one might be able to determine whether thymus lymphocytes do indeed settle and multiply in the spleen of neonatally thymectomized mice subsequently grafted with thymus tissue.

Experiments on the transfer of immunological capacity might help to test the theory that immunologically competent cells are removed by neonatal thymectomy. If such cells are absent one might expect that the capacity for an immunological response could not be transferred to appropriate hosts by means of lymphoid cells obtained from tissues of neonatally thymectomized donors, and that such lymphoid cells would not be capable of initiating a graft-versus-host syndrome in neonatal hosts, F1 hybrid hosts or lethally irradiated adult mice. If, on the other hand, transfer of immunological capacity proved to be possible one would have to envisage the possibility that cells capable of immunological reaction are present in neonatally thymectomized mice but require some non-cellular factor from the thymus in order to acquire full immunological competence. Experiments along these lines would seem to be rewarding.

The question of a non-cellular factor from the thymus has interested leukaemia students and endocrinologists for many years
(e.g. Miller, 1962). Metcalf (1958) has postulated the existence of a lymphocytosis-stimulating factor in the thymus which appears to exert some influence on lymphopoiesis. On the other hand, there appears to be some evidence that thyroid hormone may act as a specific lymphoid tissue stimulant (Gyllensten, 1959). In our laboratory we have found that radioactive iodine uptake by the thyroid of neonatally thymectomized mice is extremely low, suggesting that thyroid function may be depressed, and that some feedback mechanism may operate between the thymus and thyroid or pituitary.

**Summary**

Thymectomy of the newborn mouse is associated with severe depletion of the lymphocyte population and serious defects in the immunological capabilities of the mature animal. Such mice show striking atrophy of the spleen and lymph nodes and marked deficiency of germinal centres and plasma cells and many die from a syndrome very similar to the runting syndrome. The survival of allogeneic skin grafts is prolonged by two or more months even in strain combinations which differ at the H-2 locus and some of the mice even fail to reject rat skin grafts.

It is suggested that the thymus during foetal life and even in postnatal life produces the originators of immunologically competent cells which mature and migrate to other sites.

**Acknowledgements**

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Medawar: Is there nothing in your evidence so far to exclude a hormonal interpretation?

Miller: Nothing at all.

Medawar: You were talking about experiments in which you hoped to decide whether or not thymocytes as such would repopulate a thymectomized mouse. Even if they did, would that exclude a hormonal interpretation?

Miller: Not necessarily. A humoral factor from the thymus could

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conceivably stimulate lymphocytopoiesis in either host-type or donor-type lymphoid cells.

**Barrett:** Do you have any data yet on whether the thymocytes do repopulate?

**Miller:** Not yet. We are hoping to obtain these data by performing cytological analyses on cells dividing in the spleen of thymectomized and thymus-grafted mice of appropriate strains.

**Krohn:** Speaking for endocrinologists, I am delighted that we have at last found a function for the thymus.

In relation to the thyroid changes following thymectomy, is it possible that operative trauma might interfere with the thyroid?

**Miller:** When we do sham thymectomy we employ the same operative technique as for total thymectomy, except, of course, that the thymic lobes are not sucked out. Radioactive iodine uptake by the thyroids of sham-thymectomized mice appeared to be within the normal range.

**G. Klein:** If I understood it correctly, you could re-establish the reactivity of the thymectomized mouse by implanting an isologous thymus in some cases.

**Miller:** In most cases.

**G. Klein:** Did you try homologous, or shall we say, allogeneic thymus?

**Miller:** I have no results on that.

**G. Klein:** It would be interesting, if it succeeded, to see whether the reactivity would correspond to the donor or the host-type cell.

**Voisin:** Did you study the globulins in the sera of the animals?

**Miller:** I am trying to do electrophoresis of these sera now.

**Billingham:** Have you tried thymectomy at different ages?

**Miller:** Not in sufficient numbers to be able to establish exactly at what age thymectomy is still associated with impairment of homograft immunity.

**Billingham:** What is the latest age at which thymectomy will give evidence of graft tolerance?

**Miller:** We don’t get it after 2 weeks; I shouldn’t think we would get it after 7 days.

**Brent:** Is it possible to re-equip these mice with reactivity by using disrupted thymus cells—extracts of one kind or another?
Miller: This is what we are trying at the moment.

Hildemann: I was most interested in Dr. Miller's finding that immunologically unresponsive thymectomized mice showed a marked deficiency of lymphocytes in peripheral blood. In this connexion, in studying the ontogeny of leucocytes in newly-hatched bullfrog tadpoles, we have found that all definitive types of leucocyte appear during an age when animals may be made completely tolerant toward skin homografts—with one exception, small lymphocytes, which appear at the very time (40 to 50 days post-hatching) when the tadpoles develop the capacity to reject skin homografts. Now admittedly this is just circumstantial evidence for the function of these small lymphocytes, but it is of interest that these results are quite consistent with your view that the unresponsiveness you have observed is associated with the paucity of lymphocytes.

Loutit: I wonder if there is anything in your evidence which would suggest that the thymus is not a half-way house for cells on their way from the bone marrow to the lymphoid tissue. I have some speculations in press (Loutit, J. F. [1962]. Irradiation of Mice and Men. Chicago: Chicago University Press) which your work may show to be wrong. The speculations were based on results obtained by my colleagues, Ford and Micklem, in the adult mouse, irradiated lethally and then restored with bone marrow, syngeneic or allogeneic (preferably syngeneic) with a marker. When the mouse recovers, the marker appears, not only in the bone marrow, but in the thymus and in the lymphoid tissue. Ford and Micklem (unpublished) have given similar mice syngeneic marked bone marrow plus marked lymphoid cells. The lymphoid cells' marker appeared only in the lymph nodes, not in the thymus nor in the bone marrow; but the bone marrow cells' marker appeared in the bone marrow and the thymus. That is the early finding. After a month or two, the marked lymphoid cells disappeared from the lymphoid tissues and the bone-marrow marker appeared throughout. So that it looks as if the lymphoid cells repopulate the true lymphoid tissues initially but not permanently. The bone marrow, we considered from this evidence, contained polyvalent stem cells which repopulated the bone marrow, and the bone marrow repopulated the thymus. Cells of the marrow that were differentiating towards lymphoid cells probably circulated to the thymus, had a temporary period of residence
there, then passed on and repopulated the lymphoid tissue. Is there anything in your evidence that would shoot down that hypothesis?

**Miller:** I don’t think so. The barrier that I mentioned has been shown to exist only in normal adult animals. It is possible that after irradiation the barrier temporarily breaks down, and during this time the bone marrow cells would get to the thymus area, repopulate it and later go out again.

**Woodruff:** Would it be worth trying to re-equip these animals with one or more thymuses isolated in diffusion chambers? It might cast some light on whether or not endocrine factors are concerned in your phenomenon.

**Miller:** Unfortunately the thymus doesn’t seem to grow very well in diffusion chambers, and if you have negative results you don’t know whether it is because the thymus could not establish itself in the chamber or function properly, or whether it is because there is no humoral element from the thymus.

**Woodruff:** This prompts me to ask about the structure of the thymus in these young mice. Is there an appreciable epithelial element?

**Miller:** Yes, there is an epithelial component. There are some very large cells, which look like reticulum cells, but which have a pinker cytoplasm, and these are probably the remains of the original thymus epithelium from which the thymus develops.

**Brent:** It might be interesting to try and re-equip your mice with the thymuses taken from tolerant animals—animals made tolerant by intravenous injection of allogeneic cells at birth. You could then discover whether the animals have restored to them a general reactivity which also applies to skin grafts from the strain with whose cells the thymus donors had originally been made tolerant, or whether you get restoration of general reactivity minus reactivity towards those antigens with which the thymus donor had been injected.

**Miller:** I think the thymectomized newborn animal offers unlimited potentialities for experimental work.

**G. Klein:** Can you restore the reactivity of thymectomized mice with spleen cells from adult syngeneic mice or must you have a thymus?

**Miller:** The reactivity of thymectomized mice can be restored by spleen or lymph node cells of adult syngeneic mice in the absence of a thymus graft.
Hildemann: If the immune response capacity of these thymectomized mice is so seriously impaired that they are unable to make antibodies against bacteria and viruses, one wonders why they don't all promptly die from infectious diseases.

Miller: The mortality is extremely heavy and, at about 4 to 6 months of age, 70 per cent of mice have died, possibly from infection. Neona tally thymectomized mice may have some antibody-forming cells or other means of combating infection by viruses, but they appear to be incapable of reacting immunologically with antigenic substances that appear in the higher forms of life, such as tissue histocompatibility antigens.

Barnes: Were you repopulating only with infantile or neonatal thymuses, or did you use adult thymuses?

Miller: I always repopulate with infantile thymuses. I don't really know at what stage, in the life of the donor, thymuses will no longer be capable of repopulating when grafted to neonatally thymectomized mice.

Billingham: Dr. Miller, earlier you asked what happened to the thymuses in runt disease, and I mentioned in our analysis in the rat, when they die at 14-16 days of age of the acute form, the thymus is just a few fibrotic remnants, but I failed to state that there was tremendous hyperplasia of all the lymph nodes in the body, and the spleen; you get enlargement by a factor of 10. This mystified us considerably and I would value your opinion on this.

Miller: I can only speculate. It is possible that the thymus is overactive in this case and is trying to put out as many lymphoid cells as possible to repopulate those areas which are destroyed. And in the course of doing so exhaustion atrophy takes place.

Medawar: Do you or Dr. Loutit know if any experiments analogous to spleen shielding during whole-body irradiation have been done on the thymus?

Loutit: Not analogous, because with spleen shielding you exteriorize the spleen, and pack it round with lead. I know of no way of exteriorizing the thymus and packing it with lead. If you just take the intact animal and put a lump of lead over the thymic area you protect other things besides the thymus—the sternum and so on.

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(1956. Cancer Res., 16, 426) have done experiments in which they thymectomized mice before irradiation and then grafted thymuses from syngeneic mice subcutaneously after total body irradiation. Lymphoid tumours arose in the grafted thymus and behaved genetically as if derived from lineal descendants of thymus cells which regenerated from the donor thymus and which had not, therefore, been exposed to direct irradiation.

Medawar: Does the effect of thymectomy on susceptibility to leukaemia suggest a hormonal interpretation or a cellular interpretation?

Miller: By using cytologically marked cells, it has been impossible to uphold the theory that the thymic cells, themselves, are the cells which are transformed to leukaemic cells. This is because, in at least 80 per cent of cases of virus-induced leukaemias (Miller, J. F. A. P. [1962]. Ciba Found. Symp. Tumour Viruses of Murine Origin, p. 262. London: Churchill) or of spontaneous leukaemias (Law, L. W. [1952]. J. nat. Cancer Inst., 12, 789), it is the host cells which undergo the leukaemic change, presumably after colonizing the thymus graft. This certainly would suggest a humoral interpretation or some sort of induction mechanism.

G. Klein: I wonder whether any of your animals survived long enough for you to observe the formation of spontaneous tumours in them. I am asking this because it has been postulated recently by Burnet, by Prehn, and by us that one of the functions of the homograft reaction may be to eliminate antigenically foreign neoplastic clones that keep arising all the time. Several of the tumours induced by chemical carcinogens have been shown to be strongly antigenic in the syngeneic and in the autochthonous host, while several of the spontaneous tumours, such as mammary carcinomas, have not. There is some reason to believe that the chemical carcinogens may hamper the homograft response, and that is perhaps the reason why some of the tumours induced by them are antigenic, and why the antigenic clones can grow out. It appears to me that if the thymectomized mice lack homograft reactivity, then tumours arising in them spontaneously, which are not known to be antigenic otherwise, should be antigenic in the autochthonous and the syngeneic host, if the concept is correct.

Miller: That is a very interesting point. We are trying to get pathogen-free mice, hoping that they will live as long as possible after
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neonatal thymectomy, with a view to comparing the incidence of tumours in such animals and their controls. If the thymus is producing the originators of immunologically competent cells which play a part in homograft reactivity against antigenically distinct clones of tumour cells that arise spontaneously, then thymus regression, just before middle age, might be correlated with the higher incidence of tumours that occurs in the second half of life.

Medawar: There has been some very recently published work on the injection of antigens into the thymus itself. Hasn’t someone demonstrated plasma cell transformation in the thymus as a result of the local injection of antigen into the thymus?

Miller: Yes. These experiments were done by A. H. E. Marshall and R. G. White (1961. Brit. J. exp. Path., 42, 379). If antigen is injected intravenously there is no evidence of antibody formation inside the thymus itself. If, however, the thymus is injured by cautery or if the antigen is injected directly into the thymus, then there are histological reactions suggesting that antibody formation actually takes place in the adult thymus. It looks as if the thymus has some cells in it which are already immunologically competent, but they normally don’t produce antibodies because the antigen doesn’t get there.

Mitchison: But of what organ is this not true? If you inject antigens directly into an organ, you usually observe formation of plasma cells in it.

Medawar: It depends upon in what numbers they are produced. The interesting thing here is that the systemic injection of antigen doesn’t excite the thymus at all.

Mitchison: It doesn’t excite the skin either. But the skin is potentially capable of producing antibodies, as Oakley has shown.

Brent: Surely, in Oakley’s experiments immunologically competent cells were attracted to and trapped at the site of the antigen, and this possibility can be ruled out here by the very fact that the thymus appears to have a barrier.

Medawar: If the thymus isn’t on the pathway of lymphoid circulation, as Gowans’ experiments suggest, then this more obvious interpretation can’t be true.
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Brent: I wonder if we could discuss the idea that in order for tolerance to be operative, there must be some measure of graft-versus-host reaction. It seems to me that there are a number of instances in which it can be shown very clearly that it is possible to have tolerance without any overt signs of a graft-against-host reaction. One example is the injection of F₁ hybrid cells into newborn animals. Another example is the injection of embryonic tissues into animals, or Dr. Hašek’s parabiosis between chicken embryos. In such instances it is impossible to invoke the hypothesis which Dr. Nakić favours and on which I think his whole interpretation of tolerance in adult parabionts depends. I think we might well discuss this a little further.

Nakić: I should like to make a distinction between overt signs of graft-versus-host reaction (clinical manifestations of the disease) and microscopic findings. We may have tolerant parabiotic animals which show no clinical signs of graft-versus-host reaction whatsoever, but if you analyse the spleens or the lymph nodes of these animals you will always find "blotting-out" of the normal structure. I would like to know whether anyone has ever seen a tolerant animal where homologous immunologically competent cells have been used to induce tolerance, in which the spleen and lymph nodes do not show at least some derangement from the normal.

Medawar: I do not think that has really answered Dr. Brent’s question. You are asking if there is ever an absence of runt disease symptoms when you inject immunologically competent cells. Dr. Brent’s question turns on the injection of cells which are not competent.

Billingham: Dr. Brent’s argument is very compelling to me.
Unfortunately we do not have any measure of the reactivity on the part of the graft against the host required to get an expression of tolerance.

Woodruff: I agree. But I think it is worth making the point that graft-against-host reaction per se can weaken immunological reactivity. Howard and I (Howard, J. G. and Woodruff, M. F. A. [1961]. Proc. roy. Soc. B., 154, 532) reported some work on this last year in which graft-against-host disease was induced by injecting parent-line spleen cells into F₁’s without any radiation and where one could demonstrate impaired reactivity both towards skin grafts and a salmonella antigen. One of our two parent strains produced severe, and the other relatively mild, graft-versus-host disease in the hybrid, and the general impairment of immunological reactivity corresponded more or less to the severity of the symptoms of graft-versus-host disease. So while I would agree that tolerance can occur without any element of graft-versus-host reaction, I can imagine situations in which this additional factor might tip the scales as to whether you are going to get tolerance or not.

Simonsen: I would like to add one more case to Dr. Brent’s list of arguments—a point which I think is particularly pertinent to Dr. Nakic’s experiments—the fact that tolerance can be induced in adult parental strains parabiosed with F₁ hybrids, in which case the animal to become tolerant is not subject to graft-versus-host reaction. I would like to know, Dr. Nakic, if you think competitive replacement also occurs in these animals, and if you have any evidence of it.

Nakic: I have no experience with F₁ hybrid cells. I want to point out, however, that in contrast to dead antigen, these cells are living, self-replicating units capable of absorbing a lot of antibody. Recently Cudkowitz from Oak Ridge reported that at least in some strain combinations, F₁ hybrid spleen cells could be capable of reacting immunologically against parental tissue.

Brent: With regard to Prof. Woodruff’s point, I certainly agree
that graft-versus-host reactions can be a contributory factor. I think Castermans’ experiments show that very clearly. He repeatedly injected large numbers of immunologically competent cells into adult animals, and they eventually overruled the host response and, by reacting against the host antigens, brought about the death of the animals. What is more, animals in this state turned out to be tolerant of the donor antigens. This point seems to be clearly an example of host depletion or debilitation.

**Hildemann:** Perhaps the best example of full tolerance without graft-versus-host reactions derives from Nature’s regular experiment in natural parabiosis with multiple non-identical embryos in cattle and sheep. Here we find a long-term exchange of cells between animals in utero. The resulting chimerism and tolerance usually persist indefinitely in postnatal life and so far as I know there is no evidence that these animals suffer any immunological impairment whatsoever.

**Nakić:** I gathered from Dr. Brent and from Dr. Hašek that they did not analyse histologically the spleens or lymph nodes of stable tolerant chimeras so we still don’t know whether or not lymphoid organs of these animals show any differences from the normal.

I would like to go back to Dr. Brent’s point about using embryonic cells to induce tolerance in embryos. I agree that in several strain combinations tolerance induced with embryonic cells is not accompanied by overt signs of graft-versus-host reaction. This is used as a proof that donor cells being embryonic are capable of acquiring tolerance and are therefore incapable of reacting against the host.

I have, however, quite a different explanation for this phenomenon: it has been shown by several workers (Cock and Simonsen in chickens, Billingham and Brent in mice) that it is very difficult to induce fatal runting in animals older than 20 days; we have had a similar experience with rats. Incidence of fatal runting is very low even in mice 10 days old. I think that embryonic donor cells have to mature before they become capable of reacting against the
host; the same is true of embryonic host cells. The maturation process may take 1–2 weeks, and by this time the host may be well out of the danger period.

Billingham: In our earlier work Dr. Brent and I did find it very difficult to destroy tolerant mice immunologically by inoculation with donor-strain lymphoid cells. High dosages of homologous spleen cells were ineffective. But Dr. Silvers and I have looked into this again, using either F₁ hybrid genetically tolerant adult mice or immunologically tolerant mice. With the CBA and A combinations if you use as few as about 50 or 100 million regional node cells, then it is not difficult to kill these mice quite promptly. In other words, what spleen cells will do to a newborn mouse, adult node cells seem to do fairly well, in relatively low dosage, to adult mice. I think this is something to take into consideration.

Voisin: It seems clear to me that in some cases replacement has something to do with the establishment of tolerance, and I have been sympathetic to this concept for several years. However, one is obliged to recognize that there are some discrepancies between this concept and some observations on tolerance. On the other hand, we all know that there are a few discrepancies with the classical concepts of immunological tolerance—they are not very numerous, but I think they are highly significant. The only way I can think of to reconcile the two views is to think in terms of immunological enhancement. I feel strongly that most of the discrepancies which are observed either in the replacement theory or in the classical views on immunological tolerance to living cells can be explained on the basis of a reciprocal double-headed reaction between the host and the injected cells when these are immunologically competent—the double-headed reaction being, as I said this morning, due to serum antibodies on the one hand, leading to enhancement (or, at a higher titre, to cytotoxic activity); and, on the other hand, to cellular reactivity, more or less of the type of delayed hypersensitivity. I think that with slight modifications and adaptations (which might concern mainly selective
adaptation), this could explain all the discrepancies which have been observed.

Medawar: Dr. Voisin, could you give the evidence that has led you to believe that tolerance of cellular antigens is a totally different phenomenon from tolerance of serum proteins, as in Richard Smith's experiments, for example? I am not saying that they aren’t different, but I would like to know your reasons for thinking they are.

Voisin: It seems to me that it would make things clearer if the contrast was made, not between cellular antigens and serum proteins, but between living cells and chemically defined antigens. It then becomes easier to visualize that tolerance of living cells is probably a phenomenon different from and more complex than tolerance of chemically defined antigens.

The main fact that led me to think that this must be different is that when we use living cells they have to survive in order to induce tolerance. Of course chemically defined antigens have to “survive”, that is to stay, in the animal, in order to maintain tolerance, but the cells injected in order to induce tolerance do more than act as a permanent source of antigens; most usually, they are either immunologically competent cells or cells capable of becoming immunologically competent (and I quite agree with Dr. Nakić when, speaking of injection of embryonic cells, he supposes that these cells will become immunologically competent after a few days spent in the host). The cells usually react against the host, and when the reaction takes the form of a homograft rejection then it results in runting. Now, since we usually inject immunologically competent cells, since these cells are homologous, and able to react against the host, why do they not always react against the host? Why is it so often apparently harmless to the host, if not because there is something protecting the host against this immunological rejection reaction? In the first days of life this protective activity cannot come from a reaction of the host because this immature host is still unable to fight, so to speak, the
injected cells. This protective activity must then come from the injected cells themselves. The only mechanism I can think of for this protection is that the injected cells themselves react by producing antibodies against the newborn, and that, at a certain level, this antibody production will act as an enhancing factor and prevent the rejection reaction, or protect the newborn against the consequences of this rejection reaction. This becomes more and more a real possibility in view of the recent knowledge on competition between serum antibodies and delayed hypersensitivity.

**Brent:** I would like to ask our expert on delayed hypersensitivity, Dr. Lawrence, a question. Is a cell capable of giving the "cellular" response at the same time as it gives the serum response? In other words, can these two responses be elicited at one and the same time, or must one succeed the other?

**Lawrence:** The difficulty in giving a clear answer to the question stems from the lack of an *in vitro* test for delayed hypersensitivity and, so far, only an *in vivo* skin test to reveal its presence. Where it has been looked for in relation to serum antibody formation, delayed allergy may precede the appearance of detectable serum antibody. Our complete ignorance of the cell or cells involved in the induction, manifestations and transfer of delayed allergy would also engender caution. The lymphocyte has assumed a position of prominence in each phase of delayed allergy, but it may be more realistic to leave the matter open at this time. The answer is that we do not know.

**Brent:** Prof. Medawar and I have done an experiment in which we injected allogeneic F₁ lymphoid cells into adult mice. These normally have a strongly sensitizing effect, but when we injected them repeatedly we gradually produced a condition which very much resembled that of desensitization—reduced responsiveness to skin grafts in the presence of circulating serum antibodies. This suggested that the response of the animals, which had initially been that of a delayed sensitivity, had gradually changed over to
a circulating serum-borne antibody response at the expense of the cellular homograft response. I don't know whether Dr. Voisin would agree that his interpretation of his own experiments and those of Dr. Nakić demands that the "cellular" and the "serum" responses can occur side by side.

Voisin: I see your point, Dr. Brent. Yes, in the classical immunological reaction to a single chemically defined antigen, delayed hypersensitivity comes first and circulating antibodies come afterwards at a time when delayed hypersensitivity disappears, a sequence of events which does not support my tentative interpretation of tolerance to living cells by a mechanism of enhancement. As a matter of fact, in order to make this interpretation likely, one has to believe that a sufficient level of enhancing antibodies must be present early enough in comparison to the "rejection reaction" (which is, in your mind, bound to delayed hypersensitivity). This might be attained by at least two ways in the tolerant animals: either the phase of delayed hypersensitivity is extremely transitory, displaced by antibody production, or delayed hypersensitivity and antibody production are dealt with by two different groups of lymphoid tissue cells competing with each other. Whatever the final answer may be, it seems that this problem is part of the question of the interrelations between humoral antibodies and sensitized cells, a question with a fascinating future.

Hašek: May we return to Dr. Brent’s question about the importance of the delayed and immediate types of immunity, and their possible interactions. I think that the results with induction of tolerance may throw some light on this question as well as on the development of these two reaction pathways. Using tolerance of protein antigen in guinea pigs, J. L. Turk and J. H. Humphrey (1961. *Immunology*, 4, 310) could not dissociate the delayed and immediate types of antibody formation to a single antigen from each other.

Dissociation is known to occur between tolerance to erythro-
cytes and skin grafts (Hašek, M. and Hort, J. [1960]. *Nature (Lond.)*, **186**, 985; Štark, O., Křen, V. and Frenzl, B. [1961]. *Nature (Lond.)*, **190**, 291); however, this might be due to the fact that these two types of response are not induced by completely identical antigens.

The other question is that it is extremely difficult to calculate an equivalent dose of replicating antigen for inducing tolerance in newborn and adult animals. The immaturity of the newborn, non-reactive recipient provides an excellent proliferative advantage to the inoculated living cells whose actual dose is therefore of less significance here, whereas in an adult host an “equivalent” inoculum (per unit body weight, for example) cannot multiply (at such a rate at least) for it is reduced by immune elimination. For example, such a quantity of fresh blood as 1 per cent of the recipient’s body weight can induce tolerance in newborn ducklings both to skin graft and erythrocytes whereas in adult ducks 10 per cent body weight of blood induces tolerance to erythrocytes but is insufficient to do so with regard to skin (Hašek, M. and Puza, A. [1962]. *Folia biol. (Prague)*, **8**, 54).

In other words, the economy with a given dose of reproducing antigen is quite different in newborn and adult recipients.

* * *

*Medawar:* When I opened this conference, I made some remarks about terminology. One of the remarks I made was that it might be desirable to use a non-committal term like “promotion” to refer to prolongations of the life of homografts brought about by immunologically specific but otherwise unknown means. Someone has since pointed out that the term “promotion” is preempted for the behaviour of tumour cells, so I am afraid “promotion” is ruled out. I was attracted by the term “facilitation”, but it is too late to persuade anybody to use that now.

I don’t want to review the transactions of the conference, but I would like to say just a word about the study of antigens. In an absolute sense nothing very spectacular has emerged so far from
the study of antigens but, relatively speaking, I think progress has been very satisfactory. Two years ago it would have been unthinkable to use the words "homogeneity" or "purity" in speaking of transplantation antigens, but those words were used several times here by Davies and Kandutsch—though admittedly in a very tentative way. Obviously, a good deal of exchange of thought, and perhaps also exchange of materials, will have to take place before one can sort out the relationship between sensitizing or enhancing antigens so far made manifest by their serological activity alone.

During the meeting two wholly new conceptions have been put before us: one of them of the crucial importance of the thymus in the development of immunological reactivity; the other raising the possibility that newborn mice can be sensitized, and leading to all kinds of reflections on the relationship between tolerance and paralysis. I am quite sure you don’t wish me to summarize the rest of the meeting. We have heard a mass of important information, backed up by some pretty close reasoning. I think it fair to say that we are making rapid progress in the solution of transplantation problems. When this meeting began I said that causes for self-congratulation and self-reproach were about equally balanced, but I think that for a few weeks after this conference we might allow the balance to tilt slightly in favour of self-congratulation.

I would like to thank all of you very much for your extremely strict time-keeping and the liveliness and the pointedness of the discussion, and I would like particularly to thank the non-English-speaking contributors for the efforts they have made to deliver extremely clearly set out and perfectly intelligible papers in a foreign language. And, finally, may I thank the Ciba Foundation on your behalf not merely for making this meeting possible, but for making it possible in surroundings so conducive to thought and to enjoyment.
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*Numbers in bold type indicate a contribution in the form of a paper; numbers in plain type refer to contributions to the discussions.*

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*Author and subject indexes prepared by Mr. William Hill.*

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