The original presentation of the thesis has been amended in the following ways:

1. The word "affinity" has been replaced by "avidity" throughout.
2. When referring to cell concentrations M has been replaced by 10^6.
3. An extra reference (Ref. 20) has been added to chapter 1.
4. Figure 1 on page 32 has been elaborated.
5. On page 94 the word "only" has been erased.
6. The volume eluted in mls has been added to figure 6 on page 95.
7. Notes on figure 6 and table 3 in chapter 3 have been added on page 96.
8. The appendix after page 115 has been expanded.

Studies in Transplantation Immunity, with Special Reference

to the Induction of Specific Unresponsiveness towards

Skin Allografts in Mice Treated with Antigen,

Bordetella Pertussis, and Antilymphocyte Serum

by

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A thesis submitted for the degree of

Doctor of Philosophy

University of London   September, 1975

ABSTRACT

In the laboratory of Professor Brent and co-workers, a treatment has been developed which prolongs the survival of H-2 incompatible donor skin-allografts on immunocompetent adult mice. Specific and, in many cases, long-lasting unresponsiveness towards allografts is induced in the hosts by a combination of antigen, Bordetella pertussis vaccine and ALS treatment. This work describes several approaches investigating the mechanism of unresponsiveness.

The cell-mediated immunity of the host mice was studied using the popliteal lymph node assay, in which stimulation by allogeneic lymphoid cells injected into the footpad is measured by the increase in lymph node weight. It has been shown that mice made unresponsive to allografts retain the ability to recognise donor strain antigen in a graft-versus-host situation in an F_1 hybrid recipient, but not in a host-versus-graft situation, when F_1 cells were injected into the footpads of unresponsive hosts. Attempts to inhibit the reaction of normal lymphocytes in the popliteal lymph node assay with either cells or serum from unresponsive mice are reported; only in the former case was significant inhibition achieved.

The nature of serum factors was investigated, using the lymphocyte "homing" test of Lance. Although haemagglutination and complement fixation tests were negative, the homing test showed the presence of low titres of antigen-binding antibody in the serum. The test has been applied to ion-exchange and G200 fractions of unresponsive serum, and the results suggest that IgG and possibly IgA antibodies are involved. It was also shown that

these antibodies require the action of C_3 at least in part for the manifestation of homing activity. Experiments suggesting that these antibodies are of low avidity are also reported.

On the basis of this and other work performed in this laboratory, the mechanism of this unresponsiveness is discussed.

ACKNOWLEDGEMENTS

CONTENTS

The Reprints of Two Publications are included at the end of the Thesis

Abbreviations

Ag	Antigen
AGG	Aggregated
ALS	Antilymphocyte Serum
B.pert	Bordetella pertussis vaccine
BSA	Bovine Serum Albumin
CFA	Complete Freunds Adjuvant
CML	Cell-Mediated Lympholysis
DEAE	Di-Ethyl-Amino-Ethyl
FCS	Foetal Calf Serum
FUdR	Fluoro deoxy Uridine
GvH	Graft-versus-Host
HEPES	N-2 Hydroxyethyl piperazine-N'-2-Ethane sulphonic acid
HvG	Host-versus-Graft
HYP	Hyperimmune Serum
IFA	Incomplete Freunds Adjuvant
Ig	Immunoglobulin
ip	intraperitoneal
IUdR	Iodo-deoxy-Uridine
iv	intravenous
MLC	Mixed Lymphocyte Culture
MST	Median Survival Time
NMS	Normal Mouse Serum
PC	Pannet Compton's Solution
PLNA	Popliteal Lymph Node Assay
SAS	Saturated Ammonium Sulphate
TRIS	Tris (hydroxymethyl) methylamine
URS	Unresponsive Serum

INTRODUCTION

Only the induction of specific unresponsiveness is likely to solve the immunological problems of organ transplantation. In recent years it has become apparent that techniques of matching unrelated donor and recipient tissues, by serological or other methods, are as yet wholly inadequate; even if a perfect system of typing were to be developed, the supply of compatible donors is likely to be strictly limited. A measure of success has been achieved in renal transplantation only by the use of a prolonged course of immunosuppressive drugs, which greatly increases the risk of infection and has a number of highly undesirable side-effects. A clinical procedure is badly needed which will depress the immune response towards those foreign antigens possessed by the donor graft while otherwise maintaining the defence mechanism intact.

This specific unresponsiveness was recognised classically as being attainable in two different ways, either by depletion or deactivation of a specific clone of immunocompetent cells (tolerance),[1] or by production of antibodies which prevent contact between antigen and responder cells (enhancement).[2] This concept of "tolerance versus enhancement" proved very helpful to the immunologist but also created difficulties because some systems of enhancement appeared to have characteristics very similar to the criteria used for defining tolerance. The distinction between the two situations arose from the observation of two naturally occurring forms of unresponsiveness - unresponsiveness towards self-antigens and towards tumour growths. These phenomena have numerous contrasting features. Tolerance towards self-antigens, for example, is a state induced very early on in the development of the immune system,

before it has fully matured. The clone of antigen-sensitive cells is either absent, undifferentiated or inactivated, and the suppressive mechanism acts on the central branch of the immune system and is antigen-mediated. Enhancement of allogeneic tumour growth, on the other hand, is mediated by antibody or complexes and operates in the presence of a mature, fully competent immune system.

The clear distinction which was originally thought to exist between tolerance and enhancement has blurred over the years. Notably, the induction of unresponsiveness to histocompatibility antigens by the creation of neonatal chimeras has been shown to involve either mechanism, depending on the dose of antigen administered, and the situation where humoral or "blocking factors" operate in these animals has been called 'partial tolerance'.[3] In another system, in which survival of allogeneic rat kidneys is prolonged by enhancement with alloantibody, French and Batchelor[4] have proposed that a complex-mediated blocking mechanism must also exist. This resembles tolerance in that the inhibition has a central component. According to Baldwin and colleagues[5] antigen also may cause central inhibition in immunity to syngeneic tumour transplants.

Attempts to protect allografts by passive enhancement with injected antibody have met with varying degrees of success depending on the species, the type of organ, the antigenic disparity and the type of antibody used. In the rat, Batchelor and co-workers[6] have demonstrated considerable prolongation of kidney survival after injection of small amounts of alloantibody at the time of grafting, and similar findings have been reported for heart allografts.[7] Skin grafts, however, are notoriously difficult to enhance.

Classical tolerance, on the other hand, is a state which, once attained, is equally effective with regard to all types of donor graft. An animal which has been made chimeric by injection of semi-allogeneic bone marrow cells in early life[8,9] will retain both skin and kidney transplants indefinitely without any further treatment, and the state of unresponsiveness cannot be broken by challenge with extra antigen. In mice, tolerance is most easily achieved by injection of cells one day after birth.[10] Thereafter tolerance induction becomes progressively more difficult the older the animal,[11] and after one week is usually unattainable except in very weak combinations, or by using repeated doses of tolerogen.

In clinical situations the injection of live allogeneic lymphoid cells is highly undesirable, because of the possibility of graft-versus-host disease. Consequently, attempts to induce tolerance in adults have centred around the use of antigen tissue extracts or bone marrow cells in combination with other immuno-suppressants.

The most useful experimental system for the investigation of transplantation immunity involves inbred strains of mice or rats, because here the degree of compatibility between donor and recipient is known precisely (although this model is far removed from the human outbred situation). Mice have the additional advantage that their small size permits large numbers of animals to be used in each experiment so that the problem of biological variability can be more easily overcome. The most convenient transplant model is the skin graft,[12] for this is simple to apply, is relatively untraumatic to the recipients and can easily be assayed for rejection; it is also an extremely sensitive indicator of incompatibility.

In a series of careful experiments Lance and Medawar[13] have demonstrated

that tolerance induction in adult mice, receiving a dose of live F_1 hybrid cells,

could be potentiated by the use of Anti-Lymphocyte-Serum (ALS). In recent

years many other workers[14,15] have used ALS to deplete the blood circulation

of lymphocytes in experimental animals and so depress the immune response non-

specifically. The advantage of this form of treatment over drug therapy is that

it is non-toxic and has no side-effects if used in moderation. A single

injection of ALS is most beneficial when administered shortly after the time of

grafting, for this is the time when the graft is most vulnerable to attack by the

immune defence mechanism. Only in exceptional circumstances, however, will

such treatment cause a graft to remain indefinitely, and most regimes require

repeated treatment over a long period of time to achieve long-term prolongation.

To avoid the use of hybrid cells, for which there is no counterpart in

man, Brent and Kilshaw[16] investigated the possibility of using ALS to induce

tolerance with tissue extracts instead of live cells. The extract was prepared

from liver or spleen according to the method of Medawar,[17] and was injected

i.v. in a dose equivalent to 250 mg of fresh tissue. Crude semi-soluble

membrane extracts were used because it was considered important that there

should be no serious loss of antigenic specificities during preparation. Recent

comparative studies showed that crude extracts were more successful than highly

purified soluble preparations in producing unresponsiveness. The crude liver

preparation was much less immunogenic than the spleen extract, presumably

because of the action of enzymes released during disruption of the liver cells.

Using a course of three i.p. injections of 0.5 ml ALS, two, four and

six days after grafting, Brent and Kilshaw investigated the effect of different

time intervals between administration of extract and application of the graft. They concluded that for a "strong" combination (e.g. A —> CBA) best results were obtained with an interval of 16 days, which gave an MST of 120 days in one experiment - a survival time which cannot be explained merely by the additive effects of the two individual methods of treatment. Furthermore, they showed that retention of the skin allografts was specific, unresponsiveness not being conferred towards Balb/c or C57 grafts by an A-strain extract. The effect was strongly dose-dependent. Although some immunological activity was revealed by histological examination of the lymph nodes shortly after extract administration, it was evident that the immunogenicity of the extracts was not a crucial factor, because both liver and spleen preparations gave roughly the same degree of unresponsiveness.[18]

Because of some early findings, viz. the strain specificity, the absence of detectable antibodies and the break in unresponsiveness on injection of presensitized lymphoid cells, the mechanism originally proposed for the unresponsiveness was based on the concept of classical tolerance - the deletion of a specific clone of cells. The synergistic action of extract and ALS was thought to arise through the killing-off of antigen-specific cells which had been mobilised into the recirculating pool of lymphocytes as a result of stimulation by antigenic extract. This reasoning led to a modification in the procedure for induction of unresponsiveness,[19] in which a single injection of Bordetella pertussis causing lymphocytosis was introduced two days before grafting, in order to increase the effectiveness of the ALS. This modification led to an improvement in the median survival times for the skin grafts in the strong combination, as well as in the proportion of long surviving grafts, but in other

Figure 1   Treatment Used to Induce Unresponsiveness Towards Skin
               Grafts in Adult Mice

Treatment	Extract	B.pert.	Skin graft	ALS	ALS	ALS
Dose	250 mg eq.	0.2 ml		0.5 ml	0.5 ml	0.5 ml
Days	-16	-2	0	2	4	6

respects, the properties of the unresponsiveness were completely unchanged. The treatment used for all further experiments (incorporating the B. pertussis injection) is shown in Fig. 1.

In accord with the hypothesis of tolerance, no lymphocytotoxic, haemagglutinating or macrophage cytophilic antibodies were shown to be present at any stage of the treatment, or after grafting.[20] Nor could such antibodies be raised in these animals by a schedule of hyperimmunisation with spleen cells. Neither splenectomy nor thymectomy impaired the induction of unresponsiveness, but this state could be quickly broken by the injection of sensitised spleen cells. A second graft from the same donor strain was accepted. The presence of the graft itself was found to play an important part in the induction and maintenance of the unresponsiveness. If the grafting stage was postponed from day 0 until day 6, after the animals had received their last dose of ALS, the prolongation of survival was greatly reduced. Furthermore, if a long-surviving graft was removed and the unresponsive mouse left for more than one week before a new donor strain graft was applied, the second was quickly rejected. Hence the persistence of the unresponsive state was strongly dependent on the continued presence of antigen in the system.

However, the finding that spleen cells from mice carrying long-surviving grafts could give a normal GvH splenomegaly response towards donor antigen cast considerable doubts on the idea that this state of unresponsiveness adhered to the classical view of tolerance. A further discrepancy was demonstrated by the difference in behaviour between tolerant and unresponsive mice upon parabiosis with normal syngeneic mice.[21] Parabiosis failed to break the unresponsiveness of antigen-pertussis-ALS treated mice, and in fact, transient unresponsiveness was

transferred to the normal partners which, after separation, retained donor skin

grafts for 22 days, compared with an MST for normal mice of 10 days.

Neonatally tolerant mice, on the other hand, rejected their grafts rapidly after

parabiosis, and upon separation, their normal partners were capable of

accelerated donor strain graft rejection, presumably as a result of sensitisation

by contact with the tolerant chimera.   Unresponsiveness could be broken,

however, by treatment with cyclophosphamide, suggesting that retention of the

graft is dependent on the active participation of the immune defence mechanism.

The regime of treatment described above for inducing specific, long-

lasting unresponsiveness in adult mice has also been shown to work in rats,

and to protect heart allografts, and is in principle applicable to the clinical

situation.   It is a strictly limited treatment, not extending further than six

days after grafting, uses apparentlyirinocuous agents to bring about substantial

effects, and avoids the possibility of graft-versus-host complications.   The

extraction procedure is simple to perform, and results in a preparation of low

immunogenicity.   In order to improve on the treatment, however, it is helpful

to understand the mechanism by which this unresponsiveness operates, and the

work presented in the following chapters has been carried out with this aim

in mind.

Chapter I

STUDIES OF SERUM-MEDIATED FACTORS, EMPLOYING THE HOMING TEST

Introduction

In the homing test of Lance,[1] the ability of lymphoid cells to 'home' back to their parent lymphoid organs after reinjection into the blood circulation is measured by labelling the cells with ^{51}Cr, and then counting the radio-activity in the lymphoid organs up to 24 hours after i.v. injection.[20] The species most commonly employed are the mouse and the rat. The scheme of the test is outlined in Diagram I.

The distribution of activity among the various lymphoid organs is dependent upon a number of factors.

1)   Origin of lymphocytes

On the basis of his work with syngeneic lymph node cells,[2] Lance proposed empirically that there are two populations, defined by their differing behaviour in the homing test; – one, a lymph node seeking population, and the other, a spleen seeking population. Thus he showed that cells from the spleen home preferentially to the spleen, and lymph node cells to the lymph node. Furthermore, using anti-lymphocyte serum to deplete the recirculating population of cells in the lymphocyte donor, he established that spleen seeking cells are sessile whilst lymph node-seeking cells recirculate.[3,4] It is thought that the latter group are only transitory inhabitants of lymph nodes. Autoradiography shows that these cells

Diagram 1   The Active Homing Test

Donor   Recipient

Lymphoid
Cells

Wash, then inject i.v.

30 min incubation
with Cr-51

24 hrs later
count Cr-51 in
spleen, liver and
lymph nodes
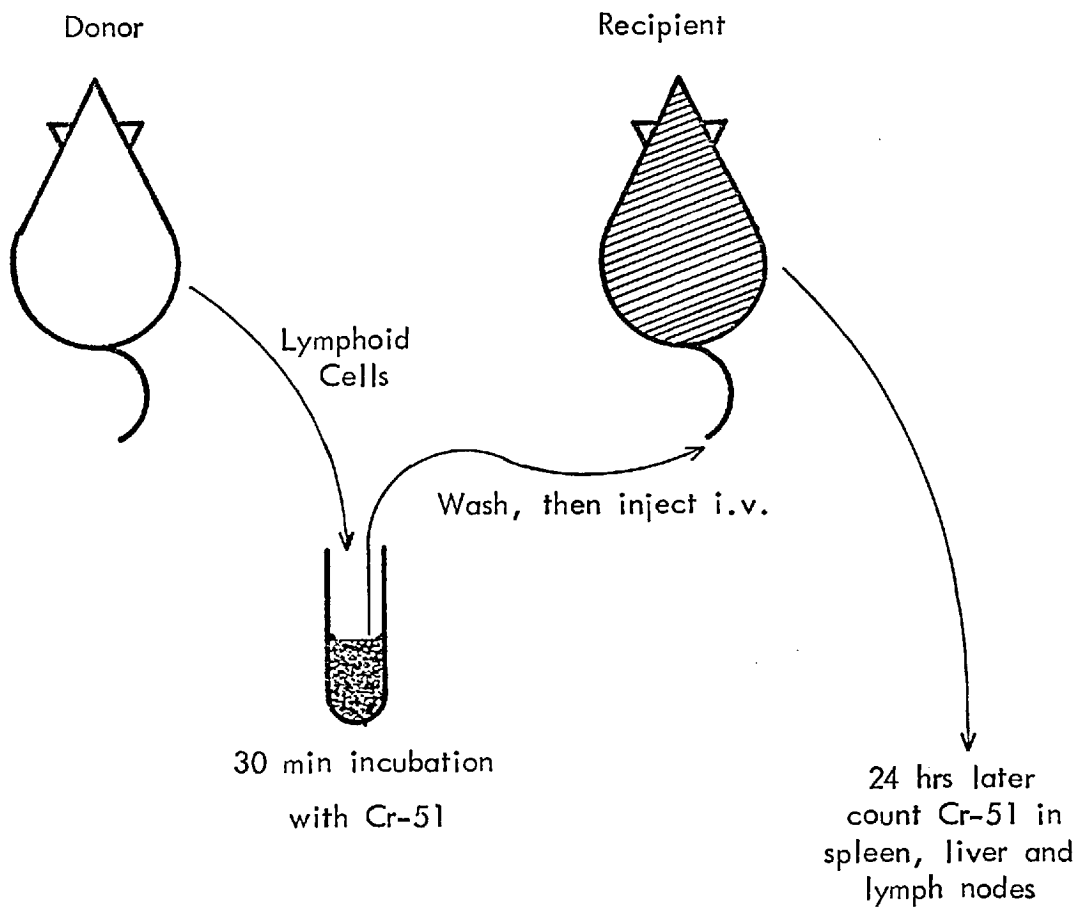
Table 1   Expected distribution of Chromium 51-labelled strain A lymphoid
          cells among organs of recipient CBA mice,   expressed as a
          percentage of the total cells injected

Treatment of cells and/or recipient	Percentage Counts in Organs				
	Liver	Spleen	Lymph Node	Total Recovered	Liver/Spleen
1 Normal distribution of syngeneic lymph node cells	20	20	10	50	1
2 Distribution of cells of low viability	50	5	5	60	10
3 Host recognition of allogeneic lymph node cells	25	20	5	50	1.2
4 Splenic trapping of syngeneic LNC induced by preinjection of SRBC	20	25	5	50	0.8
5 Distribution of syngeneic cells lacking sialic acid residues	28	20	2	50	1.4
6 Distribution of allogeneic cells in hyperimmune recipients	60	3	2	65	20
7 Distribution of syngeneic thymus cells	40	10	1	50	4.0

Diagram II   Expected time course of uptake of Chromium 51-labelled lymph node cells after i.v. injection into

normal syngeneic mice   (after Zatz and Lance[2])

Radioactive
  counts/min

in Organs

Spleen

Liver

Lymph Node

0   12   24

Time after Injection of ^{51}Cr-labelled Cells   (Hours)
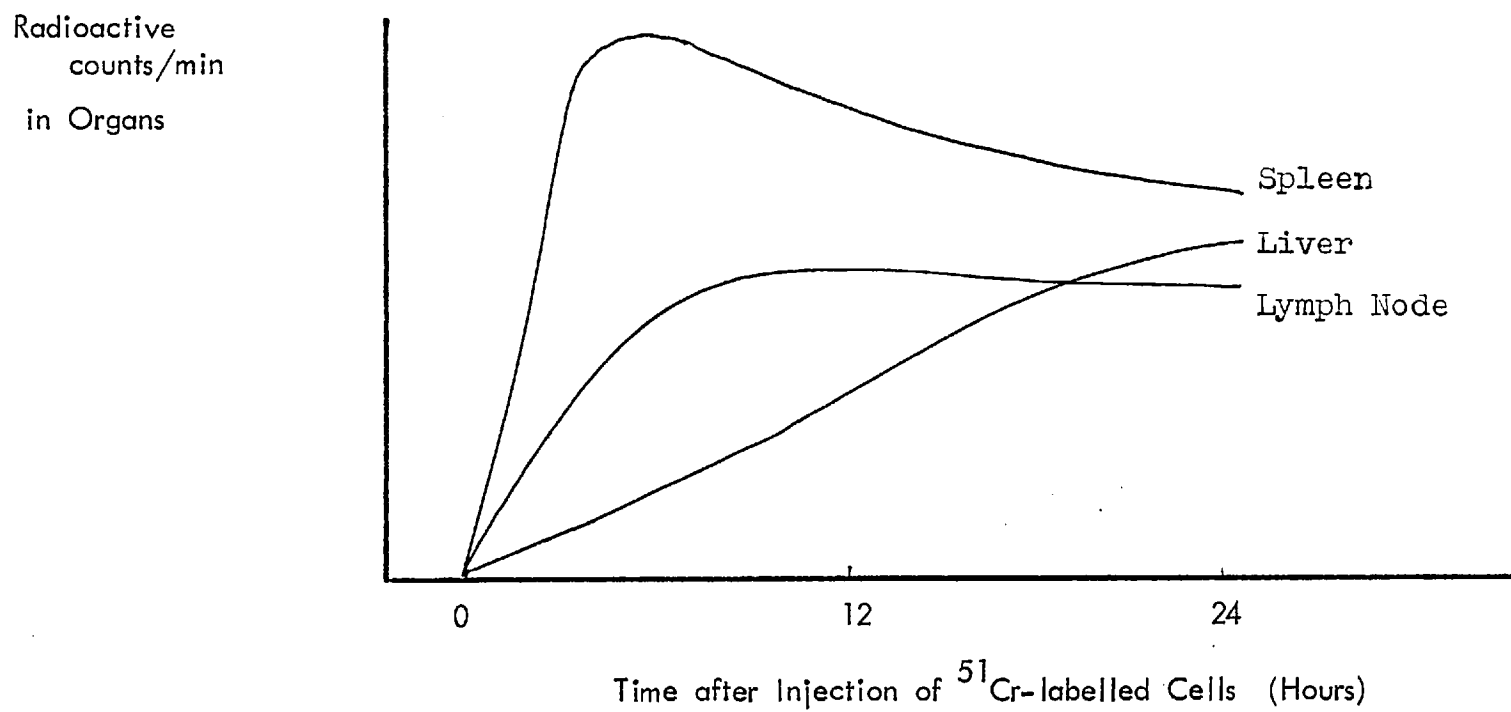
accumulate in the paracortical areas of the lymph node.[5] By labelling the rapidly proliferating cells only with [125]IUdR, Taub[6] concluded that the recirculating cells were long-lived, in contrast to the short-lived spleen-seeking cells. It should be pointed out that the spleen and lymph node retain both types of cell to a greater or lesser extent. A typical pattern for homing of lymph node cells into syngeneic recipients is shown in the first entry in Table I.

It can be seen that only about 50% of injected cells are recovered in the three organs taken, the remaining activity being distributed evenly in the circulation, lungs etc. Data for the recovery of activity from the lymph node varies between different laboratories, and depends on the particular strains of mice used, the thoroughness with which all the lymph nodes are removed, and the time after injection at which the organs are taken. A stylized diagram representing the time course of label uptake by lymphoid organs after injection of ^{51}Cr-labelled cells is shown in Diagram II. The slow decrease in activity in spleen and lymph node after 24 hours may be due to spontaneous release of chromium from intact cells. Chromium is not cleared immediately from the liver, but is reduced to approximately 50% of its initial value one week after injection.

2)   Viability

The lower the viability of the donor cell suspension, the greater the radioactivity recovered from the liver.[7] Correspondingly less is found in the lymph nodes and spleen. A certain percentage of activity is found in the liver even with suspensions of very high viability, but this may be the result of extra-cellular chromium released from live cells. The mechanism leading to accumulation of activity in the liver is not known. In Table I it is

seen that the percentage of total activity recovered is higher for low viability cell suspensions than for normal cells. It could be that damaged cells are pulled out of circulation by the liver more easily than live cells, or that the dead cells lose the ability to penetrate the spleen and lymph node tissue, and therefore remain in the bloodstream, to be removed by default by the liver.

3)   Host Recognition of Foreign Histocompatibility Antigens[8]

When there is an H-2 difference between donor and normal recipient, the distribution of activity among the three major compartments - lymph node, spleen and liver - deviates from normal in that less is recovered in the lymph node, and more in the liver (see Table I). The magnitude of the deviation is small except in strong H-2 combinations. Homing to the spleen is unaffected, so it is unlikely that damage to the labelled cells is the explanation. The phenomenon is a host response, i.e. F_1 hybrid donor cells have a modified pattern in parental recipients, but not vice versa. In irradiated or tolerant hosts, syngeneic and allogeneic cells have identical homing patterns.

4)   Trapping

Administration of antigen prior to injection of lymphocytes results in increased localisation in certain lymphoid organs.[9] Injection of sheep red blood cells i.v. enhances uptake of lymphocytes by the spleen. Subcutaneous injection of tumour cells gives increased radiolabel in the draining lymph nodes - at the expense of other peripheral lymph nodes. This is a non-specific effect which presumably helps the host to bring the clone of sensitive cells into contact with the appropriate antigen, by increasing the

total number of lymphocytes in the draining organ.

5)   Sialic Acid Residues on the Cell Surface[10]

If these residues are chemically transformed by periodate

oxidation,[11] the distribution of activity is markedly altered towards the liver

at the expense of the lymph nodes. The viability apparently remains

unaltered, however, for subsequent borohydride reduction, which reverses the

process, causes the population to revert to normal behaviour.

6)   Allo-antibody in the Serum of Recipients. (Opsonization)[12]

Allogeneic donor cells, when injected into allogeneic

recipients sensitized to donor strain antigens, are diverted to the liver (see

Table I), with a corresponding decrease in cells in both the lymph node and

spleen. This behaviour is caused at least in part by coating of the allogeneic

cells with antibody (opsonization). Whether the antibody kills the cells

before their arrival in the liver is not known. Because the antibody-mediated

effect is so large, it is not known whether sensitization of the recipient

enhances the cell-mediated homing pattern of allogeneic cells described in

Section 3.

The problem as to what is the fate of antibody-coated cells (or even

normal cells) in the liver is difficult to resolve. The mere presence of radio-

label in the liver is no indication of whether the cells are dead or alive, or

even of whether the cells are present since the label can reach the liver in a

sub cellular form, and it is retained there long after the other cell materials

have been degraded, being excreted only slowly over a period of days.

Several workers have suggested that the cells remain intact, retain their

chromium, and can re-emerge from the liver. Their evidence, however, is based mainly on the fact that the recoverable label in the liver decreases with time, and their interpretation of this phenomenon is open to question.

The thymus appears to play little part in all the above phenomena. Very little activity is ever found in the thymus, and thymocytes themselves home almost exclusively to the spleen and liver. Thoracic duct lymphocytes home in much the same way as lymph node cells.

Experimental

The Active Homing Test

An experiment was performed in the laboratory of Dr Lance, to investigate the behaviour of unresponsive mice in the homing test, in which 10^7 ^{51}Cr labelled lymph node cells (A strain) were injected into each of five unresponsive CBA recipients. The results are shown in Table II, and in comparison with normal and hyperimmune mice, the pattern of distribution strongly suggests the presence of opsonins in the serum. In cases where homing to the lymph nodes and spleen is affected equally the magnitude of the deviation from the normal homing pattern can be expressed by a 'homing index', derived by taking the ratio of radioactivity recovered in the liver, to that found in the spleen alone. This index is convenient, because it allows comparison between different experiments, even though there may be variation in the age of mice, or the dose of labelled cells.

It can be seen that, by this criterion, the behaviour of long-term

Table II   The active "homing test". Fate of Chromium-51 labelled A-strain

lymph node cells 24 hours after i.v. injection into CBA male

recipients. Distributions expressed as a percentage of the total

activity recoverable

CBA recipient	% Activity in Organs			Total Recovered	Ratio Liver/Spleen
	Spleen	Liver	Lymph Node		
*Normal	17	16	9	42	0.9
*Tolerant	19	16	9	44	0.8
*Immune	5	47	1	53	9.4
Unresponsive 1	9	33	1	43	3.7
2	5	37	1	43	7.4
3	5	44	1	50	8.8
4	8	30	1	39	3.8
5	7	30	1	38	4.3

* Figures quoted are the mean of results from six animals

Tolerant CBAs received hybrid bone marrow cells neonatally

Immune CBAs were prepared by injection of 30 10^6 A strain spleen cells i.p. followed by skin grafting

Unresponsive CBA received antigen extract and antilymphocyte serum, and had carried healthy skin graft for 100 days at the time of testing. Results 1-5 refer to individual recipients

Table III   The passive "homing test". Fate of Chromium 51-labelled A-strain lymph node cells 24 hours after incubation with serum and i.v. injection into normal CBA female recipients. Distributions expressed as a percentage of the total activity recoverable

Expt.	Serum Treatment	% Activity in Organs			Total Recovered	Ratio Liver/Spleen
		Spleen	Liver	Lymph Node		
1	NMS	22	20	5	47	0.9
	HYP	7	57	0	64	8.1
	URS I	16	42	2	60	2.6*
	URS II	21	25	4	50	1.2**
2	NMS	20	31	6	57	1.5
	HYP	6	60	0	66	10.0
	URS III	15	53	3	71	3.5*
3	NMS	13	20	5	38	1.5
	HYP	6	46	0	52	7.7
	URS IV	16	24	5	45	1.5**
	URS V	14	41	6	61	2.9*
	URS VI	17	25	6	48	1.5**
4	NMS	18	30	5	53	1.7
	HYP	7	56	0	63	8.0
	URS VII	15	32	6	53	2.1**
	URS VIII	17	32	5	54	1.9**

*   Different from NMS control by two standard deviations
** No difference

NMS: normal mouse serum from 3-month old CBA male
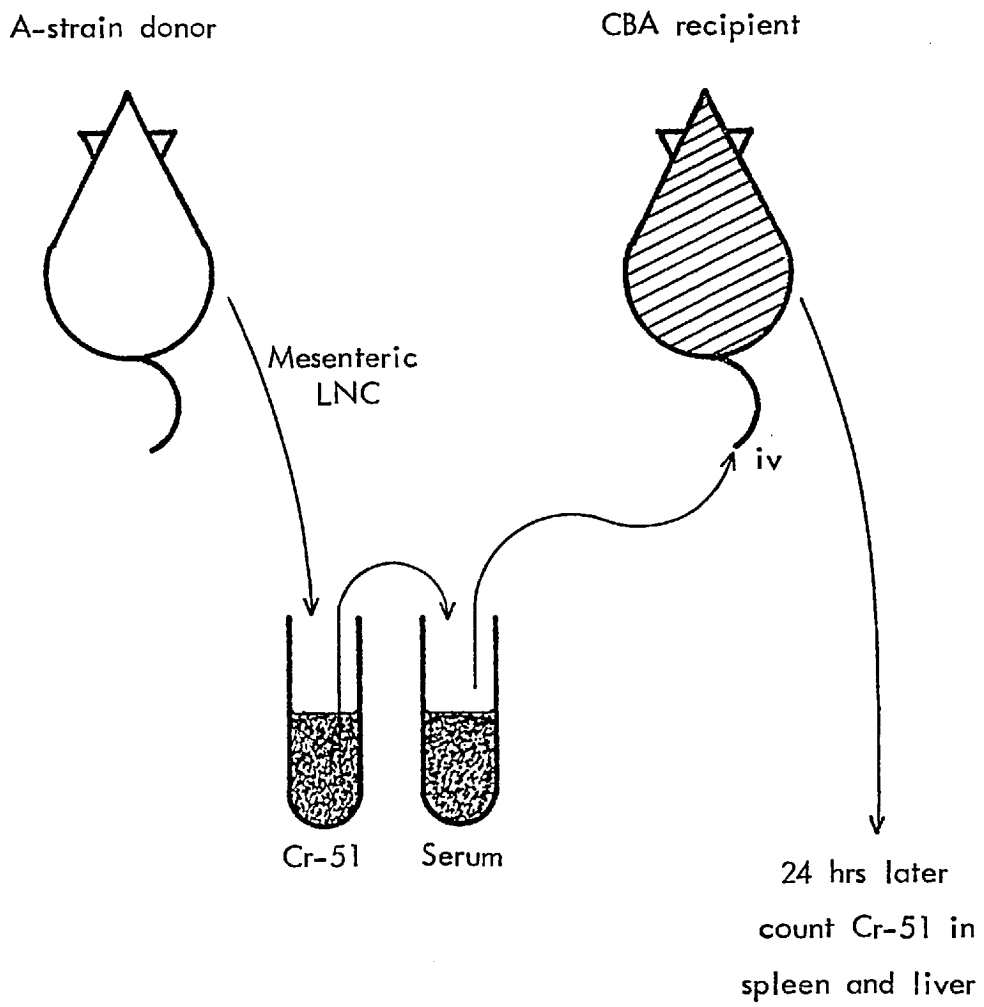HYP: hyperimmune serum raised in CBA males by injection of strain A spleen cells
URS: sera from individual CBA males made unresponsive to strain A skin grafts

All figures quoted are the mean of results from five animals

Diagram III   The Passive Homing Test

A-strain donor   CBA recipient

Mesenteric
LNC

iv

Cr-51   Serum

24 hrs later

count Cr-51 in

spleen and liver

unresponsive mice is very similar to that of hyperimmune animals with high titres of anti-A serum antibody. Other experiments show that this immune homing pattern is absent at the time of grafting, and that it becomes more pronounced with time. However, since it is known that the homing pattern can be altered by host recognition of allo-antigens even in normal recipients, more stringent tests must be applied to establish the presence of soluble serum factors.

The Passive Homing Test

A modification of the homing test was performed as follows. Mesenteric and peripheral lymph nodes from normal AO$_+$ mice were teased, and the cells were washed twice in 199 medium with 10% Foetal Calf Serum and then incubated with 200 μCi ^{51}Cr for $\frac{1}{2}$ hour at 37 deg. C at a concentration of 10^8 cells/ml, according to the normal procedure of Lance.[1] After letting the clumps settle out from suspension on standing for 5 mins, an additional step was inserted, in which the cells were incubated for $\frac{1}{2}$ hour at 37 deg. C, 10^8 cells/ml with serum at 1:3 dilution. The serum may or may not contain antibodies, which bind specifically to the labelled cells and alter their homing pattern when, after a further washing, they are injected into groups of five normal mice at a dose of 10^7 cells each. The conditions of incubation were chosen to maintain the limited amount of serum at as high a concentration as possible in suspension, without lowering the cell viability, which in all cases was greater than 70%. Table III shows the results of four experiments in which eight sera from individual unresponsive mice were tested by the passive homing technique described above. Each experiment had positive and negative controls. All mice bore perfect

grafts up to the time of bleeding (at least 100 days). The scheme of the
modified test is shown in Diagram III.

These results differ in two respects from those of the active homing test
of Lance with long term unresponsive mice as recipients (Table II). Firstly,
only a proportion of test sera (3 out of 8) show significant activity, whereas all
the test mice gave positive results. Secondly, the homing index for the three
active sera is very much lower than that for the hyperimmune serum, in contrast
to the in vivo test, in which unresponsive and hyperimmune mice have very similar
indices. These differences may arise either from some cellular participation
in vivo in the unresponsive hosts, or from the decreased sensitivity of the
modified test. It may be that working at the threshold of sensitivity the
passive test only detects the strongest sera. Cells are incubated with serum at
a 1:3 dilution for only half an hour, while in the active test the incubation
time is effectively 24 hours, with undiluted serum.

Modification of the Passive Homing Test

The sensitivity of the test was increased by decreasing the number
of cells injected per dose. The smaller number of cells involved during
incubation permits use of smaller liquid volumes, while still maintaining
adequate culture conditions. Consequently, test serum can be used neat during
incubation. Using cell doses below 10^7, not enough activity is obtained in
the lymph nodes to give a reproducible result. However, for the opsonization
test, there appears to be no advantage in using the lymph node data, and in
further experiments activity recovered from the lymph nodes is not recorded.

Table IV shows that decreasing the cell dose has no effect on the normal

Table IV The effect of cell dose on the homing index of labelled lymph

node cells in normal allogeneic recipients (A —> CBA)

No. of Cells	0.5×10^8	1×10^7	2×10^6	5×10^5
Homing Index (Liver/Spleen)	1.7	2.0	2.0	1.8

Table V   Effect on the homing index of preincubation of labelled strain A

lymph node cells with 0.4 ml neat mouse serum before injection

into normal CBA females

Incubation medium	Liver/Spleen Ratio
Washing Medium (Phosphate buffered PC)	2.3
Normal Mouse Serum	2.3
Hyperimmune Serum	13.0

Table VI   Titration of hyperimmune serum: Chromium-labelled strain A

lymph node cells were incubated with decreasing concentrations

of hyperimmune serum before injection into normal CBA females.

The sensitivity of the original and modified forms of the passive

homing test are compared

Dilution of Hyperimmune Serum	1:1	1:10	1:100	1:1000	1:10,000	MEDIUM
Modified Test**	10	11.5	6.8	3.2	2.0	1.6
Original Test*	–	5.7	2.6	1.5	–	1.4

* 5×10^7 cells incubated with serum in volume of 2 ml

** 4×10^6 cells incubated with serum in volume of 0.4 ml

homing index whatsoever. Each value is the average from five mice. Standard errors are all within $\pm$ 0.2.

Incubation of cells in 0.4 ml neat normal mouse serum has no adverse effect (no toxicity) compared with washing medium (Table V). The hyperimmune serum was, as always, inactivated by heating at $56^{\circ}C$ for 30 minutes.

In the modified test as used in later experiments, 4M labelled cells are incubated in 0.4 ml neat serum for $\frac{1}{2}$ hour. After incubation, 1 ml medium is added to the suspension and five mice are injected with 0.2 ml aliquots i.v., i.e. with 5.5×10^5 cells. Table VI compares the sensitivity of the test before and after modification, in a titration of hyperimmune serum.

In the modification, the hyperimmune serum still has activity at a concentration of 1:10,000. Haemagglutination rarely measures concentrations lower than 1: 2,000.

Dilution of Unresponsive Serum

In another experiment, a sample of pooled unresponsive sera was titrated out (Fig. 1). A 10-fold dilution of this serum pool reduces the concentration to such an extent that no activity is discernible, and this can only mean that the opsonin is present at a low concentration. That the serum factor is strain specific is demonstrated by the fact that third-party cells (in this case C57Bl) display the normal homing pattern after incubation with unresponsive serum (Table VII). This rules out the possibility of participation of residual ALS in the host.

Figure 1   Titration curves for hyperimmune and unresponsive sera in the modified passive homing test
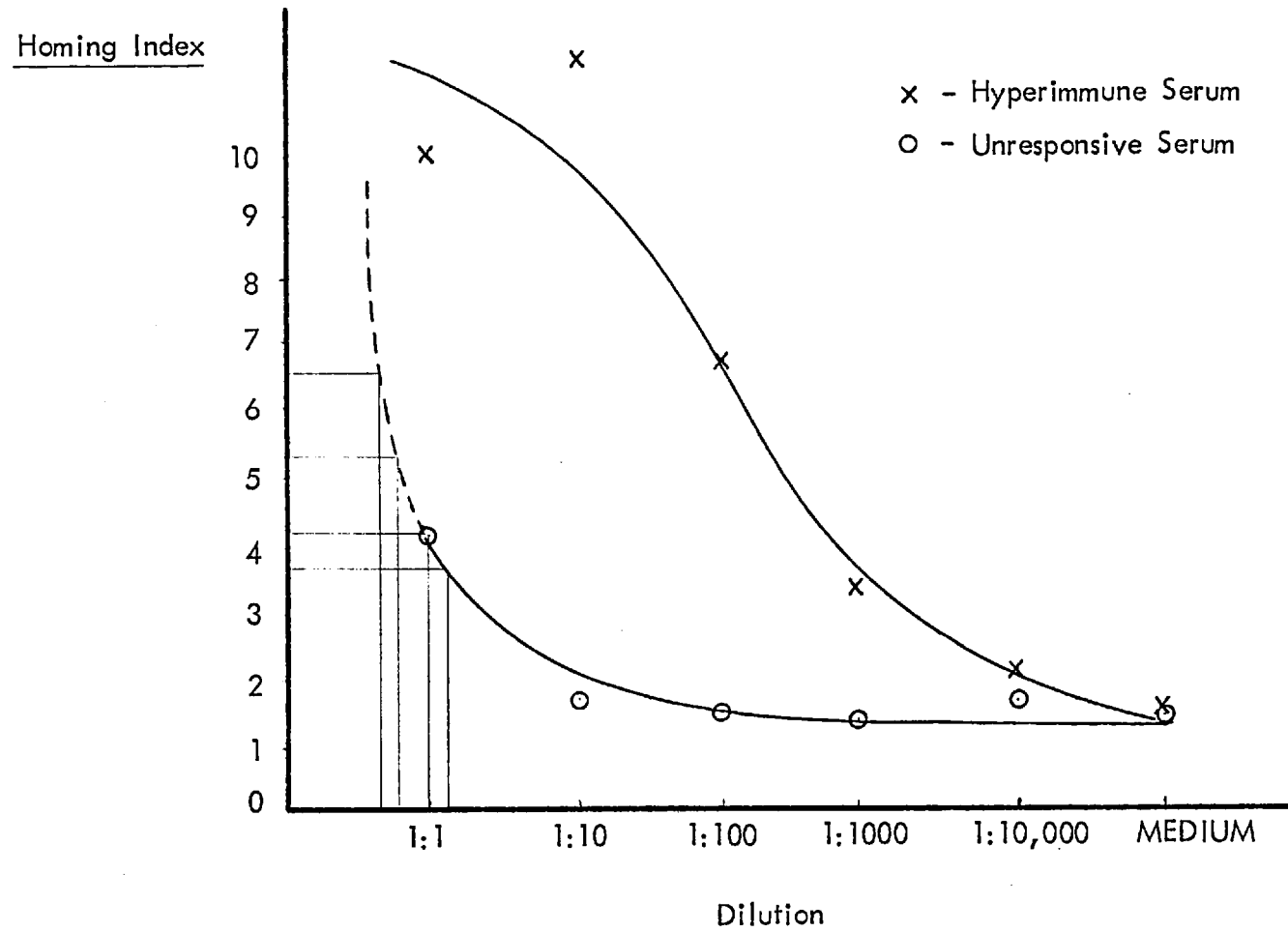
Table VII   Homing indexes of labelled third-party (C57Bl) lymph node

cells after incubation with serum from CBA males unresponsive

to A-strain skin allografts

Sera	Strain of Labelled Cells	Homing Index
HYP	A	16.3
URS	A	10.4
URS	C57Bl	0.9
MED	C57Bl	0.8

HYP: hyperimmune serum
URS: "unresponsive" serum
MED: medium (phosphate-buffered PC)

Table VIII   Homing indexes after incubation of neat sera (0.4 ml) with two

consecutive batches of 4×10^6 labelled lymph node cells.   Each

batch of cells was used in the modified passive homing test

Sera:	NMS	HYP	EXP I	EXP II
1st Incubation	1.8	17.4	6.5	4.3
2nd Incubation		17.9	5.2	3.8

NMS and HYP are Normal Mouse Serum, and Hyperimmune Serum respectively

EXP I and EXP II are "unresponsive" sera from the same pool but incubated
    on different occasions

Table IX   Homing index of labelled strain A lymph node cells in normal

CBA females: Effect of length of incubation time with serum

from "unresponsive" CBA males

	HYP	MED		URS	
	$\frac{1}{2}$ hr	$\frac{1}{2}$ hr	$\frac{1}{4}$ hr	$\frac{1}{2}$ hr	1 hr
Homing Index (Liver/Spleen)	8.3	2.3	3.1	3.3	3.5

HYP: hyperimmune serum
MED: phosphate-buffered PC
URS: "unresponsive" serum

Absorption of "Unresponsive" Serum by Cells

Table VIII shows the results of an experiment designed to find

the proportion of serum factor absorbed by cells during a half hour incubation

with serum. The experiment was carried out as described, except that after

incubation the cells were spun down and resuspended in fresh medium before

injection, and the supernatant was retained and used for a second incubation

with labelled cells, which were treated similarly, before injection into a second

group of recipients. In each case, 4×10^6 cells were incubated with 0.4 ml

neat serum (either unresponsive or hyperimmune). In this experiment, the second

batch of cells was diverted to the liver almost as much as the first batch.

Assuming that the liver/spleen index is related to the amount of surface-bound

opsonin, the fact that the initial titre is so low (compared with hyperimmune

serum) suggests that the cell surface is not completely covered. This may be

because the opsonin is of low ·avidity, or because it will only bind with a

small proportion of those cell surface antigens which hyperimmune serum will

bind with, or because steric factors permit binding only very slowly. However,

an experiment in which the incubation time was varied from $\frac{1}{4}$ hour to 1 hour

(Table IX) suggested that very little extra material was absorbed after $\frac{1}{2}$ hour,

and that most was taken up after the first 15 minutes. It seems unlikely that

the homing index would increase significantly during a longer incubation.

If the antibody is either of low avidity, or is directed towards only a

small sub-population of surface antigenic determinants, then it must be

concluded that after 30 minutes incubation with unresponsive serum the surface

of the cell is covered as fully as possible, for no more will bind. If this were

the case one should be able to attain homing indexes in the passive test for

unresponsive serum as high as those in the active test. The fact that this is not so can be explained if one proposes that after i.v. injection of antibody-coated cells the antibody is of low enough avidity to come off the cells while in the circulation. In an unresponsive animal antibody present in the serum will replace this, and the cells will remain fully coated. In the passive test, however, which uses normal recipients, there is no serum antibody. Consequently the passive test is likely to give lower homing indexes than the active homing test.

Several experiments have been done to investigate further the phenomenon of opsonization itself. To test the hypothesis that the liver/spleen ratio is a function of coverage by immunoglobulin on the cell surface, a comparison was made of the titration curves of the same hyperimmune serum with allogeneic (A-strain) and hybrid (AXCBA) F_1 lymph node cells. Because the hybrid cells have fewer alloantigens on their surface, one might expect lower titres than for fully allogeneic cells. In Fig. 11, with the exception of one anomalous point at 1:100, homing indices of allogeneic cells are consistently higher than those of hybrids. The end-point is reached sooner for hybrid cells, and even at high concentration there is some decrease in activity.

Age of Labelled Cells

A second point examined concerned the effect of age on the serum-mediated homing pattern. Zatz,[13,14] has investigated the way in which the relative proportions of spleen and lymph node seeking cells in a cell population are altered with age, and in fact there appear to be only small variations in the liver/spleen ratio (approx =1) from the age of three months

Figure II   Titration curve for hyperimmune serum incubated with either A-strain or (CBA x A) F_1 hybrid Cr-labelled

lymph node cells

Homing Index

x –   A-strain Cells

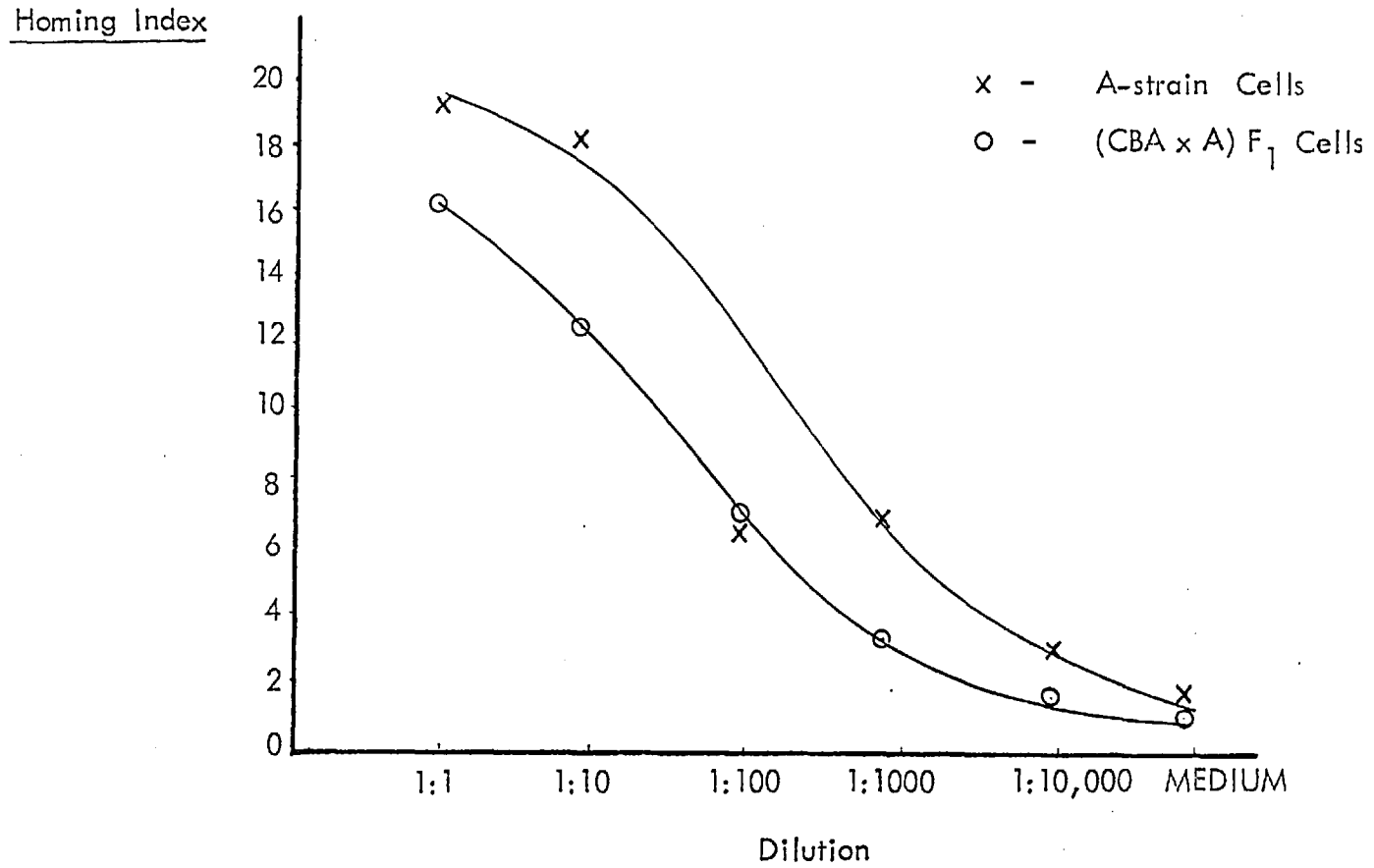o –   (CBA x A) F_1 Cells

Dilution

Table X   Homing index of labelled strain A lymph node cells in normal

CBA females following incubation with hyperimmune serum:

Effect of age of cell donors

Age	NMS	HYP
3 months	1.2	19.5
12 months	1.3	20.1

NMS: normal mouse serum
HYP: hyperimmune serum raised in CBA males against A strain spleen cells

Table XI Effect of prior in vivo C_3 depletion of recipients by Cobra Venom Factor on the distribution of radioactivity after i.v. injection into CBA females of antibody-coated strain A chromium-labelled lymph node cells

Serum	CVF	Experiment I			Experiment II		
		Activity in Organs		Ratio Liver /Spleen	Activity in Organs		Ratio Liver /Spleen
		Spleen	Liver		Spleen	Liver	
MED	−	2380	3700	1.5	2050	4170	2.0
	+	1980	3510	1.8	2100	4100	2.0
HYP	−	440	7720	17.5	280	7670	27.4
	+	870	7460	8.6	580	7160	12.3

Table XII   Effect of C$_3$ depletion in vivo by Cobra Venom Factor treatment on

the homing pattern of strain A lymph node cells incubated with

"unresponsive" serum

	Sera:	HYP	MED	URS (no treatment)	URS (CVF)
Activity in Liver		12650	8030	10200	8270
Activity in Spleen		1520	3550	3110	3160
Ratio Liver/Spleen		8.3	2.3	3.3	2.6

HYP: hyperimmune serum raised in CBA males by injection of strain A spleen cells

URS: "unresponsive" serum

Table XIII   Effect of in vivo C_3 depletion by Cobra Venom Factor treatment

on the homing pattern of labelled strain A lymph node cells

incubated with "unresponsive" serum, and with dilute hyperimmune

serum

	CVF	Activity in Liver	Activity in Spleen	Ratio Liver/Spleen
HYP (neat)	−	4520	350	13
MED	−	1930	1190	1.6
HYP (^{1}/100)	−	5010	590	8.5
	+	3960	570	7.0
URS	−	2810	780	3.5
	+	2150	830	2.5

HYP: hyperimmune serum raised in CBA males by injection of strain A
        spleen cells

MED: phosphate buffered PC

URS: "unresponsive" serum

onward. An experiment performed in this laboratory (Table X) comparing cells from old and young mice shows no difference after incubation with hyperimmune serum.

Dependence of Homing on Complement

Several experiments were performed to investigate the requirement for complement in antibody-mediated homing. C_3 levels in mice were depleted by injection, over a period of twenty four hours, of four doses of Cobra Venom Factor (CVF), to give a total of 6 units per mouse (200 units per kg). This treatment has been shown to reduce the levels of C_3 by at least 95% for up to thirty-six hours.[15] The day after CVF treatment, the mice received ^{51}Cr-labelled lymph node cells, incubated with either hyperimmune or normal mouse serum. Organs were removed for counting the following day. The sera (including FCS in the medium) were always inactivated. In Table XI, in which two such experiments are shown, the results have been recorded as the absolute magnitude of activity recovered in the liver and spleen, in order to demonstrate the relative number of cells affected by CVF treatment. This treatment has no effect on the homing of normal cells (L/S ratios 1.5 compared with 1.8). Although the liver/spleen ratios for the cells incubated with hyperimmune serum are very different, (17.5 no treatment; 8.6 CVF), this results from only a very small discrepancy in the cell numbers reaching the spleen – approximately 5% of the total counts recovered. Homing to the liver is virtually unaffected.

Effect of C_3 Activation on URS-mediated Homing

A similar experiment using unresponsive serum (Table XII) shows almost a complete reversion to the normal homing pattern as a result of CVF treatment. Here, the difference is manifested in the activity of the liver, and involves about 20% of the total counts recovered. This may simply reflect the fact that the unresponsive serum is at a lower titre than the hyperimmune. It could be that there are two mechanisms involved in antibody-mediated homing, one dependent on C_3, and one complement-independent, only those cells with large amounts of antibody on their surface being able to reach the liver in the absence of complement. On this basis, one would expect a large reduction in the homing index for unresponsive serum because this probably covers the cell surface only partially.

In a further experiment, lymphoid cells were incubated with very low titres of hyperimmune antibody, and then injected into CVF-treated mice. In this case, the distribution of cells follows the same pattern as those incubated with unresponsive serum (Table XII). The radio-activity in the spleen is altered very little, but the difference manifested in the liver involves about 20% of the total counts recovered. Thus it seems likely that the difference in behaviour of unresponsive serum is indeed due to the fact that the antibody is in low concentration.

In interpretation of these results, one must remember that CVF is a mixture of substances with a wide range of effects,[16] and that it acts to deplete C_3 by non-specific activation, causing it to undergo cleavage to C_{3a} and C_{3b}. One might wonder, therefore, whether the CVF treatment does not have a non-specific inhibitory effect on the reticulo-endothelial system, for

example causing a blockade in the liver by red cells coated with C_{3b}, and so reducing the hepatic uptake of lymphoid cells. This possibility can be discounted, however, since the homing of normal lymph node cells to the liver is not inhibited by CVF treatment. Furthermore, other workers[17] have observed no reduction of hepatic uptake of SRBC in CVF-treated mice even when the dose of red cells reached 10^8. One must conclude therefore that the differences observed are due to depletion of C_3, and not due to an increase in C_{3a} or C_{3b} levels in the circulation.

Discussion

The homing test is potentially a very powerful tool for the analysis of immune mechanisms in vivo. It traces the fate of lymphoid cells in the circulation of the subject host and observes the way in which the immune defence system alters their normal destinations. This deployment of lymphocytes by the host is an important part of any immune response, and is a phenomenon which is impossible to reproduce in vitro. By observing lymphocyte accumulation in various compartments of the host it is possible to look at different cell sub-populations, and the method has been used both as a cell analyser, and a cell separator. The test lends itself to studies of cell surface determinants, particularly those which govern the distribution of lymphocytes among the lymphoid compartments. It is possible also, to observe the cell-mediated reaction of a normal host towards transplantation antigens at a very early stage. At a later stage the test proves to be a very sensitive assay for the presence of antibodies against transplantation antigens.

There are certain difficulties in the interpretation of the homing test, mainly because the different host compartments do not take up lymphocytes independently of each other. Thus in some cases where the activity recovered in the lymph nodes is low, it is difficult to know whether this is because the penetration of lymphocytes into the nodes is more difficult than normal, or because the retention of these cells by the liver has been somehow increased. Under such circumstances perfusion experiments with isolated organs (e.g. spleen, liver and lymph node) are very useful for clarifying the issue.[18, 19] The second difficulty arises from the use of chromium as a marker for cells, because this label is not readily cleared from the liver. Consequently, it is difficult to tell whether activity in the liver is due to intact cells, to cells which have been phagocytosed, or to extra-cellular chromium spontaneously released from live cells in the circulation.

Neither of the above factors prevent the use of the homing test as a probe for immune responses, provided comparison is made taking all the relevant parameters into account. A third limitation, however, relating especially to immunity to transplantation antigen, is that there are not enough independent parameters to enable one to investigate several unrelated phenomena when they act concurrently. In particular, in a sensitised animal, the deviation from the normal homing pattern caused by cell-mediated host recognition of alloantigen may be so small that it is completely swamped by the antibody-mediated phenomenon, and in the presence of serum alloantibody remains completely undetected. For this reason, in experiments where the interest lies in the behaviour of unresponsive mice towards donor transplantation antigens, the homing test has proved an unsatisfactory bio-assay

for unresponsiveness, because the presence of soluble factors (which may not themselves have a direct bearing on the unresponsiveness) in the serum prevents it from measuring the cell-mediated immunity in these animals.

With respect to the serum factors, however, the homing test has yielded a great deal of information which was not forthcoming from the haemagglutination, Coombs, complement fixation, or macrophage adherence tests. The participation of residual anti-lymphocyte serum has been ruled out by the demonstration of specificity towards donor antigen. Also, the homing activity increases with time after grafting, whereas the concentration of anti-lymphocyte serum would decrease. The antigen-binding specificity and complement dependence of the serum factor active in the test strongly suggest that it is an antibody, although there is no formal proof. The factor appears to be at a very low concentration, as shown by dilution experiments, yet at its highest concentration only a small proportion will bind to the cells during incubation, enough being left over in the supernatant to bind a significant amount with a second batch of cells. This suggests that the antibody is either of low avidity-, or is directed towards only a small sub-population of surface antigenic determinants, (see appendix).

Finally, it must be stressed that, having established that the deviation from normal homing pattern displayed by unresponsive hosts is due to antibody, one cannot assume that this antibody is in any way responsible for unresponsiveness to allografts.

Chapter II

CELL MEDIATED IMMUNITY OF UNRESPONSIVE MICE

Introduction

The ability of lymphoid cells from unresponsive animals to show

reactivity towards donor strain antigen in vitro, in situations outside the host,

prompted the question whether these cells would behave in the same way inside

host tissues, at a time when the animal was bearing a long-surviving skin

graft. It was decided to test this in a Host-versus-Graft system (HvG) where

there existed also a Graft-versus-Host (GvH) counterpart in which reactivity

was demonstrable. The politeal lymph node weight gain assay of Ford, Burr,

and Simonsen,[1] was adopted for this purpose.

This assay is designed to quantitate the magnitude of a cell-mediated

response towards cellular antigen, by observing changes (e.g. weight, blast

cell formation) in the popliteal node draining a footpad previously injected

with lymphoid cells. The advantage of this test over other in vivo systems for

assaying cellular immunity is that it is a local one-way reaction, between

two lymphocyte populations, which takes place in an immunocompetent host.

(This is in contrast to the splenomegaly assay, where the host is either neonatal,

or irradiated, or to the Elkins test under the kidney capsule, where the site is

practically devoid of host lymphocytes.) Consequently, one can reverse the

direction of the recognition reaction without altering any other factors in the

environment contributing to, or arising from, the cell-mediated response. Thus,

in addition to the graft-versus-host response, the test has been used to

demonstrate a host-versus-graft reaction against both major and minor

histocompatibility antigens,[2,3] and against virus-induced cell surface antigens.[4]

The popliteal lymph node assay is a good model for cellular reactions

in general, because it displays many of the important features of a typical

cell mediated response. These include the following:

1)   Specific recognition, in which there is a requirement for foreign

antigenicity of the stimulating cell population. Korčáková and Hašková[5] have

separated the recognition process from later stages of the GvH reaction by

culturing the two allogeneic lymphoid cell populations in vitro, and

transferring the cell-free supernatants from these cultures into the footpads of

hybrid recipients. They have demonstrated an increase in the incidence of

activated cells in the popliteal node, assessed by morphological examination

of the nucleoli of cells in the node. Other criteria for GvH activity were

not studied, however, and although it appears unlikely, the necessity for donor

cell participation in later stages of reaction has not been ruled out. Yoshida

and Osmond[6] studied the GvH activities of various types of donor lymphoid

cell. Using cell populations from different organs, and fractions of bone-marrow

cells separated on an albumin density gradient, they observed a correlation

between activity and the proportion of small, recirculating T-lymphocytes in

the donor inoculum. This is in accord with other models of cell-mediated

immunity.[7,8,9,10]

2)   Proliferation

The GvH reaction has often been assayed by incorporation of

the thymidine analogue ^{125}IUdR into the DNA of dividing cells. Monie and

Everett,[11] used this technique to distinguish between dividing host and donor

populations with differing chromosome markers, and discovered that most of the

proliferating cells are of host origin. This presumably reflects the great

excess in the lymph node in the number of host cells over donor cells, both

of which are capable of responding non-specifically to stimulatory factors, such

as those described by Hašková. Splenomegaly brought about by GvH reactions

in F_1 neonates has also been shown to involve predominantly host proliferation.[12,13]

GvH in irradiated hosts,[14,15,16] or under the kidney capsule,[17] is a result of

donor proliferation, as is to be expected, for in both these tests the reaction

takes place in conditions where the host population is small.

3)   Influx of Cells

The major contribution to the lymph node enlargement,

observed following an antigenic stimulus, is non-specific trapping of

recirculating lymphocytes by the lymph node. Many workers[18,19] have shown

that the efferent ducts of the node are closed down, and that the output of

cells into the lymph is greatly reduced. Furthermore, in response to adjuvants

such as Bordetella pertussis, the node will expand to accommodate extra cells

prior to infiltration, even in mice which have been chronically depleted of

circulating lymphocytes.[20] Emeson and Thursh,[21] have demonstrated

[51]Cr-labelled lymphocyte trapping in popliteal nodes stimulated by GvH and

HvG reactions, as well as in the nodes draining skin grafts.

4)   Cytotoxicity

An experiment performed in this laboratory has demonstrated the

generation of cytotoxic cells in the popliteal nodes stimulated by a host-versus-graft

reaction. It has not yet been established whether the cytotoxicity (measured by in vitro ^{51}Cr-release assay) is specific. One discrepancy between the cell-mediated responses in the skin-graft and GvH models, is that the reactive populations are apparently stimulated by different sets of antigens. Oppltová and Démant[11] studied a wide range of strain combinations using the popliteal lymph node assay, and showed that responder cells in GvH (and MLR) are stimulated most by differences at the Ir sublocus, whereas skin graft rejection is independent of Ir differences, requiring only H-2K or H-2D incompatibility. (There is conflicting evidence in some cases.[23]) It should be remembered, however, that skin graft rejection is an assay for only one small subpopulation of cells (killer cells), whereas GvH and HvG reactions look at several different populations at the same time. Most people agree that the killer cells in the CML test recognise H-2K and H-2D differences only,[24] and it could be that study of cytotoxic cells evoked in the Popliteal Lymph Node Assay will bring the two models into line with each other.

Experimental Section

The experimental procedure for the popliteal lymph node assay was as follows. Groups of five mice were injected in one footpad with 20 μl of a suspension in PC of 2.5 -10x10^6 allogeneic lymphoid cells. These cells had previously been teased from mesenteric lymph nodes, washed three times, and allowed to settle for five minutes to remove clumps of dead cells. In the contralateral footpad, a dose of syngeneic cells was injected, to provide a control for non-specific factors leading to node enlargement (or otherwise) -

e.g. stimulation by the medium, cell debris, age of animals, or just the relatively large numbers of cells injected. Not more than six days later, the animals were killed, and the nodes were excised free of fat and weighed to an accuracy of $\pm$ 0.1 mg on a Oertling microbalance. The nodes were prevented from drying out by storing them on a piece of filter paper saturated with saline. An index of activity was obtained by calculating the ratio of the two lymph node weights for each animal, and then summing the ratios for all animals in the group. In this way, one compensates for individual variations in the reactivity of mice in a group.

Male animals were used for almost all experiments. In the GvH tests the host mice were 3 month old (CBAxA) F_1 hybrids unless otherwise stated. The donors were either CBAs made unresponsive to skin grafts, or normal CBAs of the same age, or normal CBAs which had received Bordetella pertussis and ALS at the same time as the unresponsive mice. Donors of syngeneic control cells were taken from the same pool as the F_1 hybrid hosts. In the HvG tests the cell suspensions were from 3 month old (CBAxA) F_1 hybrid donors, injected into CBA hosts. The treatment of these hosts varied from experiment to experiment, and their precise origin has been specified individually for each experiment in the results section of this chapter.

Following the method of Pritchard and Micklem,[25] reactivity was also assayed by incorporation of the thymidine analogue ^{125}IUdR into the DNA of dividing cells in the lymph nodes, six days after initial challenge. The animal's own pool of thymidine was reduced by administration of cold FUdR, which blocks the synthetic pathway for thymidine in vivo. At the same time, 5 μCi/animal ^{125}IUdR was injected i.v., and the activity in the lymph node was measured in a Packard Scintillation Counter 3 to 5 hours later.

Figure I

Host-versus-graft assay:

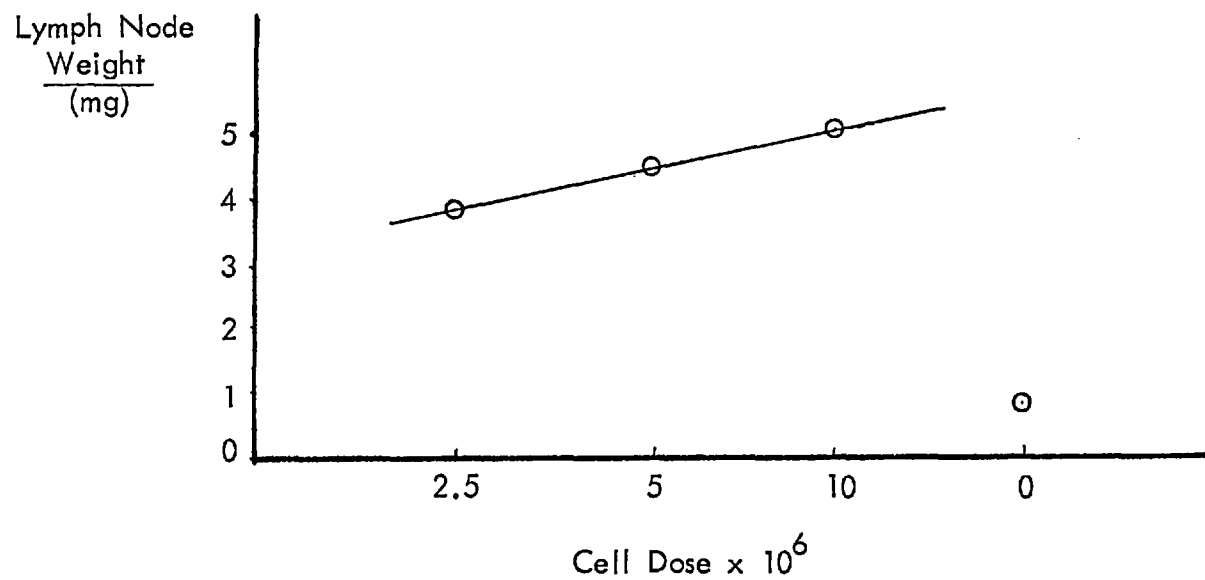Dose-response curve in Balb/c ——> CBA combination
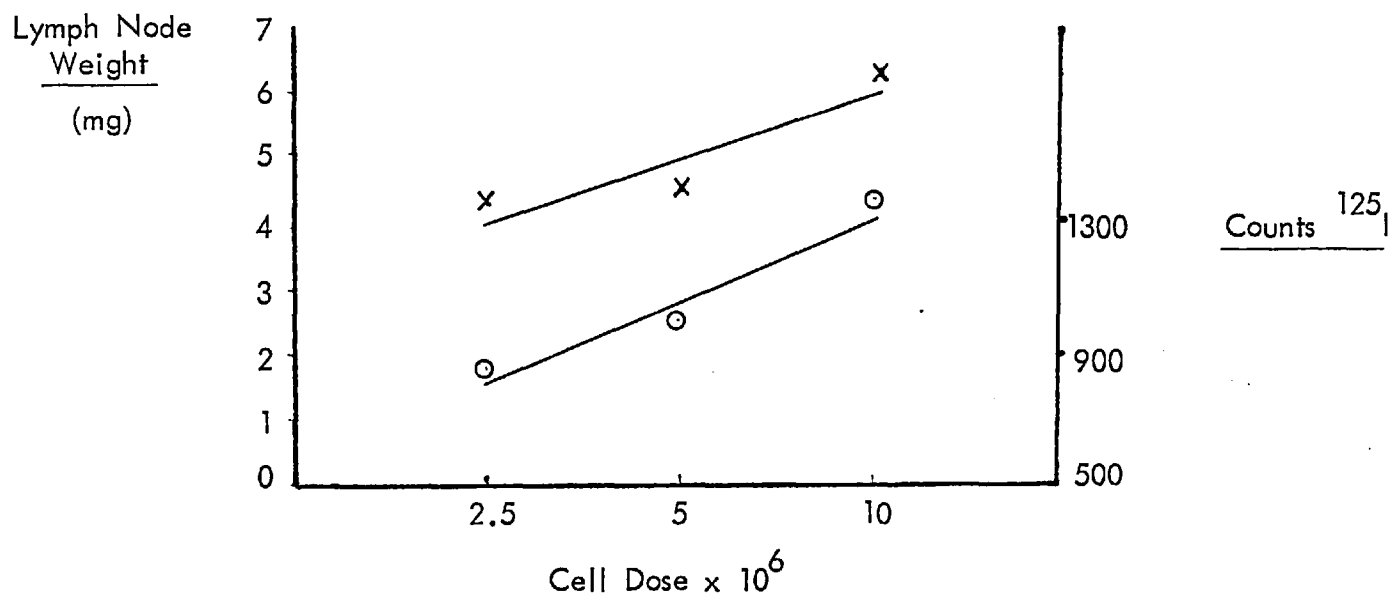
Each point on the graph represents the pooled results from five mice.

x - lymph node weight   o - ^{125}I counts

Figure II

Host-versus-graft assay:

Dose-response curve in A —> CBA combination

Lymph Node Weight (mg) vs Cell Dose x 10^6

Each point on the graph represents the mean of five lymph node weights

Table 1   Host-versus-graft assay:

Effect of dead syngeneic cells

	Footpad	% Dead Cells in Dose	Ratio ($^{right}/$left) $\pm$ S.E.
Group I	left	50	
	right	100	1.5 $\pm$ 0.1
Group II	left	75	
	right	100	1.2 $\pm$ 0.1

Hosts and donors were taken from the same pool of 3-month old CBA males. Both groups contained five mice.

Table II   Host-versus-graft assay:

Effect of washing syngeneic donor cells with Foetal Calf Serum

Host	Footpad*	Treatment of Donor Cells	Ratio Weights	(right/left) Counts
CBA	left	washed without FCS		
	right	washed with FCS	1.5 ± 0.1	3.4 ± 0.3

* injected with 5×10^6 cells

The group contained five animals.

Table III   Host-versus-graft assay:

Effect of washing of donor cells with Foetal Calf Serum on the

response to allogeneic cells

Host	Strain of Donor	FCS	Weight of node	Ratio ± S.E. Wts.	Counts
Balb/c	(Balb/c x CBA)F_1	+	3.8	1.8 ± 0.2	2.9 ± 0.4
	Balb/c	+	2.1		
	(Balb/c x CBA)F_1	−	4.0	3.2 ± 0.8	4.7 ± 0.4
	Balb/c	−	1.5		
	(Balb/c x CBA)F_1	+	4.1	1.2 ± 0.2	1.1 ± 0.2
	(Balb/c x CBA)F_1	−	3.3		
	Balb/c	+	3.3	1.6 ± 0.6	1.9 ± 0.7
	Balb/c	−	2.3		

Cell dose per footpad was 2.5×10^6 cells

Each result is the mean from five animals

Results

Figure I shows a dose response curve for an HvG experiment in which 3 month old Balb/c females were injected with (Balb/c X CBA) F_1 hybrid cells. Results for both methods of assay are shown. A dose response curve for the HvG test in the A —> CBA combination (CBA host) is presented in Figure II. The latter system is seen to be less sensitive than the Balb/c —> CBA combination. As the dose response curve is fairly flat over the 2.5 - 10×10^6 cell dose range, and as the viability of doses greater than 10^7 probably deteriorated rapidly on confinement within a 20 μl volume, most of the subsequent experiments used a dose of 5×10^6 cells/footpad.

The results of other preliminary experiments are shown in Tables I- III. The non-specific effect of dead cells was demonstrated by injecting different groups of Balb/c mice with a constant number of syngeneic cells, in which there are varying proportions of live and dead cells. Dead cells were obtained by irradiating a suspension of high viability lymph node cells in PC and 2% FCS with 2000 rads. Suspensions of 50% and 75% dead cells were obtained by mixing live and dead cell suspensions in appropriate proportions. ^{125}IUdR was used to assay the response. Both ratios were greater than unity, implying that the presence of dead cells provided non-specific stimulation of the node. As expected, the difference between 100% and 50% (group I) was greater than 100% and 75% (group II).

In the previous experiments, all washings were performed with PC supplemented with up to 10% FCS, in order to ensure maximal viability of the injected cells. To avoid injecting free FCS into the footpad, which is itself antigenic, and likely to stimulate the node the final suspension is made

up in PC alone. To investigate the effect of FCS, an experiment was performed in which two cell suspensions were prepared by washing in PC with or without 10% FCS, and these two suspensions were injected into opposite footpads of the same syngeneic hosts. Table II shows that some stimulation occurred as a result of washing in FCS. The same total number of cells was injected regardless of viability.

If the effect of the FCS was merely to prevent cell death, then, in contrast to the actual findings, group I should have stimulated less than group II, for it contained fewer dead cells. A more likely explanation is that some FCS proteins may have been introduced into the node by adherence to the surface of injected lymphocytes. To test this hypothesis fully, a dose response curve should be performed comparing different numbers of lymphocytes treated in both ways, to see if the slope of the FCS curve is steeper than that of the untreated cells. This was not done, however, because it was felt that the assay system was not sensitive enough to detect the small differences involved.

The effect of FCS on HvG responses in an H-2 incompatible combination (Table III) was to increase the stimulation produced by syngeneic control cells proportionately more than the stimulation by allogeneic hybrid cells - i.e., the non-specific background was raised. On the basis of these results, FCS was omitted during cell washing in all further HvG experiments. In GvH experiments, where viability of the donor cells is of the utmost importance, FCS was retained in the washing medium. A dose response curve in the A ⟶ CBA combination is shown for a GvH reaction in Figure III.

Figure III

Graft-versus-host assay:

Dose response curve in A $\longrightarrow$ CBA combination
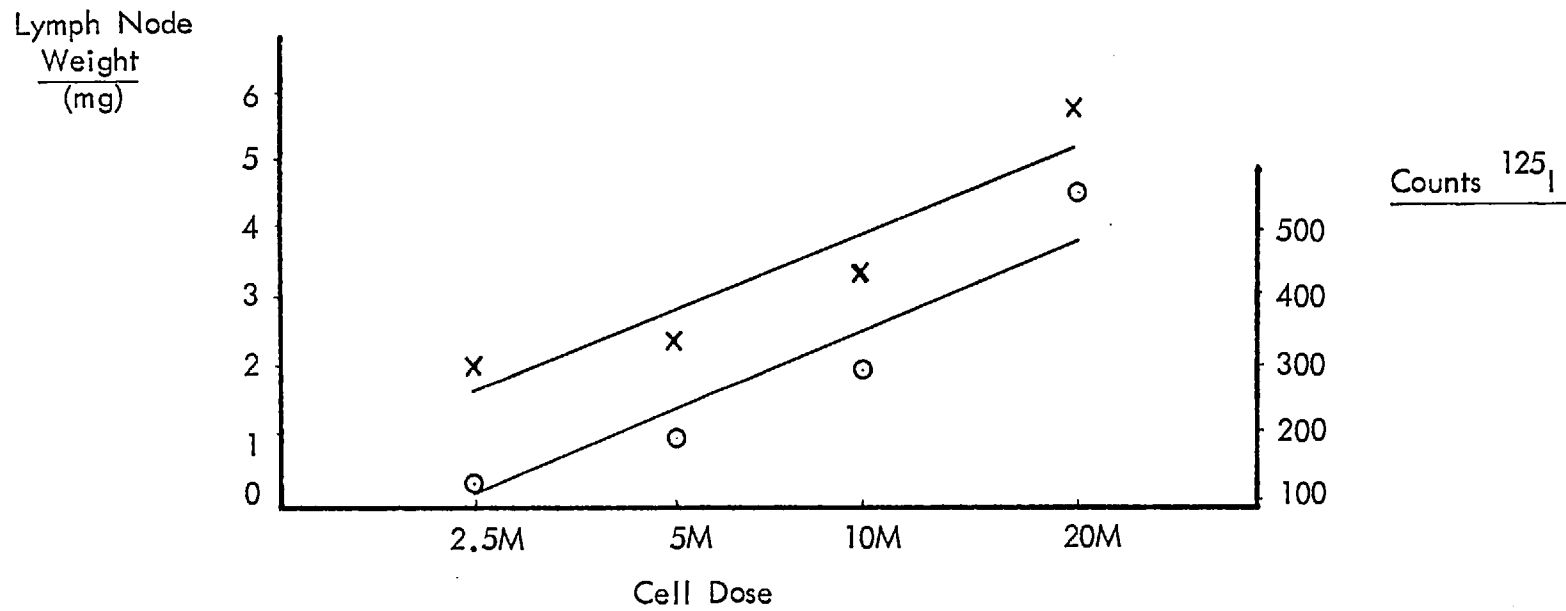
x - Lymph Node Weights

o - Counts of ^{125}I

Table IV   Reactivity of cells from "unresponsive" mice in the

graft-versus-host assay

Experiment No.	Footpad (left/right)		Ratio (left/right) $\pm$ S.E.
1	Control group	Normal/F_1	1.6 $\pm$ 0.2
	Test group	Unresp/Normal	1.4 $\pm$ 0.3
2	Control group	Normal/F_1	1.7 $\pm$ 0.2
	Test group	Unresp/F_1	2.1 $\pm$ 0.4
3	Control group	Control/F_1	2.0 $\pm$ 0.1
	Test group	Unresp/F_1	2.4 $\pm$ 0.3

Each group contained five animals

In experiments 1 and 2, recipients were (CBA x A)F_1 hybrid males, three months old. Test animals were unresponsive to A strain antigens, and had borne healthy A strain skin allografts for 80 days.

The control animals were normal 3 month old CBA males, and F_1 hybrids from the same pool as the recipients.

In experiment 3, the test mice were unresponsive towards C57Bl allografts, and consequently (CBA x C57Bl) F_1 hybrid males were used as recipients. The control animals here were donors age-matched with the unresponsive mice, and treated with Bordetella pertussis and ALS at the same time as unresponsive mice.

GvH Assay with Unresponsive Mice

The popliteal lymph node assay using the method already described was first applied to measurement of the GvH reactivity of cells from mice made unresponsive in adult life to donor strain antigens. Mesenteric lymph nodes were removed from mice that had carried healthy A-strain skin grafts for 12 weeks, teased and washed twice in 10 ml PC with 10% FCS, allowed to settle for 5 minutes to remove cell clumps, and finally injected in 5×10^6 cell doses into the footpads of 3 month old (CBA x A) F_1 hybrid males. The object of the test was to make a comparison between these cells, normally reactive CBA cells, and F_1 cells (which are genetically unreactive to F_1 antigens). The results are shown in Table IV. Whilst in experiments 1 and 2 CBA donors unresponsive to A-skin grafts were used, experiment 3 was conducted with a CBA donor which had been made unresponsive to C57Bl antigens. In the latter experiment the "control cells" were from normal age matched CBA males treated with Bordetella pertussis and ALS (but not antigen extract or skin graft) at the same time as the unresponsive animal. In all three experiments, the unresponsive cells show GvH activity at least as great as the normal cells, there being no statistically significant difference between the experimental and normal groups.

HvG Assay with Unresponsive Mice

These results are in strong contrast to those of the analogous HvG experiments (Table V), where antigenic F_1 cells provoked no response when injected into the footpads of unresponsive host mice. The experiment was repeated several times with different controls. Experiments 1 and 2 show

Table V   Non-reactivity of "unresponsive" CBA male hosts in the

host-versus-graft assay

HvG Experiment	Host*	Cells injected**		Lymph Node Wt mg	Ratio + S.E. left/right
1	Unresp. to A	l	$(CBAxA)F_1$	1.3	1.9 ± 0.5 Test
		r	–	0.9	
	Normal	l	$(CBAxA)F_1$	3.3	3.7 ± 0.7 +ve Control
		r	–	0.9	
	Normal	l	Normal	1.6	2.9 ± 0.4 –ve Control
		r	–	0.6	
2	Unresp. to C57Bl	l	$(CBAxC57Bl)F_1$	2.1	3.4 ± 0.4 Test
		r	–	0.7	
	Control	l	$(CBAxC57Bl)F_1$	3.8	4.1 ± 0.4 +ve Control
		r	–	0.9	
	Control	l	Control	2.3	2.7 ± 0.3 –ve Control
		r	–	0.9	
3	Unresp. to A	l	$(CBAxA)F_1$	2.4	0.9 ± 0.1 Test
		r	Unresp.	2.7	
	Control	l	$(CBAxA)F_1$	3.8	1.9 ± 0.2 Control
		r	Control	2.0	
4	Unresp. to A	l	$(CBAxA)F_1$		1.1 ± 0.1 Test
		r	Unresp.		
	Control	l	$(CBAxA)F_1$		1.6 ± 0.2 Control
		r	Control		

*   Each group contained five animals

**   Footpads were injected with 5×10^6 cells in 20 μl
     "Control" animals were age-matched CBA males which had received
     B.pertussis and ALS at the same time as the unresponsive CBA males.
     Normal animals were three month old untreated CBA males

Table VI   Fate of Chromium 51-labelled (CBA x A) F_1 lymph node cells

24 hours after footpad injection* in CBA males unresponsive to

strain A skin grafts

Animal No.	% Activity Recovered in Popliteal Node	
	Left F_1 cells	Right CBA cells
1	1.5	0.8
2	-	1.1
3	0.8	0.8
4	1.1	1.1
5	1.4	1.0
Mean	1.2 ± 0.2	1.0 ± 0.1

* 5×10^6 cells

the drawback of using uninjected footpads as controls: a slight variation in the small lymph node weight gives a large difference in the ratio. The third ratio in each of these two experiments demonstrates the non-specific enlargement due to injection of syngeneic cells. In both these experiments, the stimulation in unresponsive hosts is significantly less than in the positive controls. Experiment 4 was assayed using ^{125}IUdR incorporation, and lymph node weights were therefore not obtained. In the last two experiments, controls syngeneic with the host were employed (i.e. unresponsive cells into unresponsive hosts) to avoid any possible consequences of introducing reactive cells into an otherwise unresponsive host. It is seen here that the reactivity of normal control animals towards F_1 cells is completely absent in unresponsive hosts.

A possible objection to these experiments is that the lack of reactivity may be due to the removal of antigen from the lymphatics by serum factors before the antigen reaches the lymph node. The effect of serum on lymph node cells injected i.v. into unresponsive mice has already been demonstrated by homing experiments, in which the presence of opsonins diminishes the total number of cells reaching the lymph nodes. To test whether this factor is of any importance in the HvG experiments, the fate of ^{51}Cr-labelled lymph node cells was traced after injection into the footpads of unresponsive mice. (A x CBA)F_1 hybrid cells were injected into one footpad and CBA cells into the other. No difference in the quantity of labelled material remaining in the popliteal nodes was found after one day (see Table VI). Because of the shortage of unresponsive mice other times after injection were not looked at, but it is known from conventional homing experiments[26] that once cells are removed from the circulation they are relatively inaccessible to the

Table VII   Host-versus-graft assay in CBA males unresponsive to strain A grafts

Simultaneous challenge by donor (A-strain) and third-party (C57Bl) antigens

Host	Cells Injected	Ratio (l/r) $\pm$ S.E.
Unresp. to A	l. (C57Bl x A) F_1 r. Unresp.	1.6 $\pm$ 0.2
Control	l. (C57Bl x A) F_1 r. Control	2.0 $\pm$ 0.2

Each group contained five recipients.

Age-matched control CBA males had been treated with B. pertussis and ALS at the same time as the unresponsive CBA males.

opsonization effects of serum factors.

Specificity of Unresponsiveness

To test whether the unresponsiveness in HvG reactions is strain-specific, one should challenge simultaneously with two different antigens, one of which is the original donor strain antigen, the other being from an unrelated third party strain. Furthermore, since we are dealing with a local reaction, both antigens should be administered at the same site. If the unresponsiveness is specific, one would expect a reaction towards third party antigens to occur in the absence of any response to donor strain antigen. If, however, challenge by donor antigen causes a non-specific depression of the immune response, then the HvG reaction should be diminished, even towards third party antigens.

In the experiment shown in Table VII, (C57Bl x A) cells were used to present donor and third party strain antigens simultaneously to the unresponsive host. According to the genetic data of Snell and co-workers[27] on the strains C57Bl, A and CBA, with the exception of antigen 32 at the D end of the H-2 locus, CBA cells have no histocompatibility antigens which are not also possessed by either C57Bl or A strain cells. Consequently, there can be no recognition of CBA antigens by hybrid cells, and the reaction is only one way.

Table VII shows that the hybrid cells elicited comparable response in both unresponsive and Pertussis-ALS treated controls, suggesting that the unresponsiveness is indeed strain specific. Although the ratio for the unresponsive mice is lower than for the controls, one must remember that it represents a response towards an antigenic stimulus only half as great as that

in the controls. One cannot exclude the possibility, however, that the reactivity might be due to recognition by the hybrid cells of unknown antigens (perhaps LD antigen) on the CBA cells.

Inhibition by Serum

The striking difference in reactivity of lymph node cells from unresponsive hosts, depending on whether or not the cells were removed from their host, suggests the action of some active suppressor mechanism operative only in the milieu of the host. The most likely explanation is the participation of soluble factors in the serum, which inhibit the activity of cells with a normal response towards A-strain antigens. When the cells are removed from host tissues, washed and injected into a normal host, the factors are no longer present and a positive GvH results. If this were the case, then it might be possible to inhibit the activity of normal cells by injection of unresponsive serum. Table VIII shows the results of a preliminary experiment to find the relative quantities of normal serum immunoglobulin (iodinated) arriving in the popliteal node and other organs after footpad or i.v. injections of this material. The treatment resulting in most ^{125}I-Ig reaching the node is injection of 0.5 ml i.v. However, if serum factors operate by binding either to antigen, or to antigen-sensitive cells, then the success of inhibition experiments will be favoured by footpad injection, where there is less chance of these factors being absorbed out in the circulation before they reach the node. Consequently, to test the inhibitory effects of unresponsive serum on GvH, 20 μl was injected into footpads of host mice on day 0, day 1, and day 2 after injection of cells.

Table VIII   Influence of route of injection on the distribution of

^{125}I-immunoglobulin among various organs

Dose	Route of Injection	^{125}I-Counts Recovered* in			
		Popliteal node left	right	Spleen	Liver
20 μl	left footpad	130	30	700	17,560
0.1 ml	i.v.	160	120	3120	110,000
0.5 ml	i.v.	480	590	18,000	540,000

* Mean of three animals

Table IX   Inhibition of normal host-versus-graft and graft-versus-host responses

by serum from CBA mice unresponsive to A-strain skin grafts

Experiment	Host	Donor Cells	Serum	Ratio $\pm$ S.E.
GvH 1	$(CBA \times A) F_1$	CBA	URS	1.2 ± 0.1
		$(CBA \times A) F_1$	NMS	
	$(CBA \times A) F_1$	CBA	NMS	1.8 ± 0.3
		$(CBA \times A) F_1$	NMS	
GvH 2	$(CBA \times A) F_1$	CBA	URS	1.6 ± 0.2
		$(CBA \times A) F_1$	URS	
	$(CBA \times A) F_1$	CBA	Control	2.3 ± 0.4
		$(CBA \times A) F_1$	Control	
HvG	CBA	$(CBA \times A) F_1$	URS	1.6 ± 0.2
		CBA	NMS	
	CBA	$(CBA \times A) F_1$	NMS	1.9 ± 0.2
		CBA	NMS	

Each group contained at least five recipients. Cells were obtained by pooling

mesenteric lymph nodes from three donors per group.

All sera were stored at $-80^{\circ}C$ before use.

URS is serum from CBA males made unresponsive to A strain skin grafts.

Control serum comes from CBA males age matched with unresponsive mice,

and treated with Bordetella pertussis and ALS.

NMS comes from normal three-month old CBA males.

Table X   Inhibition of normal host-versus-graft response by iv

injections of serum from CBA mice unresponsive to

A-strain skin grafts

Host	Donor Cells	Serum	Ratio $\pm$ S.E.
CBA	(CBA x A) F_1 CBA	URS	5.2 $\pm$ 0.9
CBA	(CBA x A) F_1 CBA	-	7.7 $\pm$ 0.5

Each group contained five recipients. Cells were obtained by pooling

mesenteric lymph nodes from three donors per group.

URS is serum from CBA males made unresponsive to A strain skin grafts.

It was stored at -80° C before use, and was injected ip. in a single dose

of 0.5 ml on the day of cell injection.

In Table IX the results of two GvH inhibition experiments are given. In both cases, unresponsive serum appeared to reduce the stimulation due to normal cells. Experiment 2 was probably better controlled because unresponsive serum was injected into both footpads of the first group, so that any non-specific depression by URS was allowed for. The control serum was from Pertussis-ALS treated age-matched controls.

Inhibition of the HvG assay (Table IX) was less successful, possibly because of the much larger pool of antigen-sensitive cells which must be inhibited in this assay. Consequently, a further experiment was performed in which 0.5 ml unresponsive serum was introduced systemically (i.v.) into the test animal, in the hope that suppressive factors might act on the recirculating pool of antigen-sensitive cells, before they make contact with the antigen, and so prevent them from being trapped in the lymph node. Although the enlargement index turned out to be unexpectedly high in both groups, there was considerable depression of reactivity in the group treated with unresponsive serum (see Table X).

Statistical Treatment of Results

Although none of the results quoted above are individually significant at the 5% level in the Student t test, they all follow a consistent trend. Since both the experiments in the GvH and HvG series are measuring the same phenomenon, it is possible to combine the results of these experiments by fitting the raw data to a general linear model, and carry out a statistical analysis of the pooled results, with effectively a much larger number of experimental values in each sample. This was done using

the Bio Med BMDX64 general linear hypothesis program with the help of

Mrs Doreen Irving from the Statistics department at the London School of

Hygiene and Tropical Medicine. The logarithms of the original lymph node

data were pooled for the HvG and GvH tests, and an analysis of variance

(F test) was performed which showed that the "group" effect in each case was

significant at the 5% level. For the HvG experiments, $t = 2.49$ with

21 degrees of freedom and for the GvH, $t = 2.20$ with 17 degrees of freedom.

Inhibition by Cells

An alternative mechanism for active host suppression is that of

suppressor cells. In the light of recent work by Gershon,[28] and Asherson and

Zembala,[29] it was decided to investigate this possibility, again using the

popliteal lymph node technique. Attempts to inhibit the HvG reactivity of

normal cells were performed in two ways, either by mixing putative suppressor

cells with the antigenic stimulant before injection into the footpad (experiment

1, Table XI) or by injecting unresponsive cells systemically into the host by

i.p. injection (experiment 2, Table XI). In the former system, the 'suppressor'

cells were introduced directly into the site of reaction, so that the danger of

dilution is minimised. On the other hand, footpad injection limited the dose

of cells very considerably, whereas with the i.p. injection a much larger total

number of unresponsive cells was present in the animal as a whole.

As can be seen, unresponsive cells completely failed to suppress in

experiment 1, whereas the response (admittedly small) of normal cells in

experiment 2, was totally abolished by the large dose of unresponsive cells

injected i.v. These results are inconclusive, and unfortunately no further

work was possible.

Table XI Inhibition of the normal host-versus-graft response by cells from

CBA mice unresponsive to A-strain skin grafts

Experiment	Host	Dose of Cells Injected in footpad ($\times 10^6$)	i.p.		Ratio $\pm$ S.E. ($^{left}/_{right}$)
1	CBA	l. 2.5 Unresp. +2.5 F_1 r. 2.5 Unresp.	– –		4.3 $\pm$ 0.5
	CBA	l. 2.5 Control +2.5 F_1 r. 2.5 Control	– –		3.0 $\pm$ 0.3
2	CBA	l. 5 F_1 r. 5 CBA	10	Unresp.	1.1 $\pm$ 0.1
	CBA	l. 5 F_1 r. 5 CBA	10	CBA	1.5 $\pm$ 0.4

At least five animals were used in each group. Three month old normal CBA
males were recipients in all cases (n – CBA). F_1 cells were from 3 month old
(CBA x A) F_1 males. Cells denoted "unresponsive" were from mice bearing
A-skin grafts for more than 100 days. Control cells were from CBA males
age-matched with the unresponsive mice, which received Bordetella pertussis,
and ALS at the same time as the unresponsive males.

Discussion

The data from the popliteal lymph node assay has been supplemented by results from in vitro experiments carried out in this laboratory by C.G. Brooks.[30] He demonstrated that unresponsive cells have a virtually normal MLC reactivity towards donor strain antigens. Bearing in mind, however, that graft rejection is mediated by cytotoxic killer cells, he also tested populations of MLC-stimulated cells for cytotoxic activity in the Brunner cell-mediated lympholysis test (CML), and showed that unresponsive cells caused as much target cell lysis as did normal control cells after in vitro stimulation. Cytotoxic activity was also present in this population after in vivo stimulation by prior injection of $5 \times 10^7 F_1$ spleen cells i.v. into unresponsive animals. In this case, however, the cells were distinctly hyporeactive. An important point to remember is that, even after induction of cytotoxic cells by injection of donor strain spleen cells into unresponsive mice, the allograft is usually retained.

The inability of F_1 cells to produce a host-versus-graft reaction in unresponsive mice suggests that there is an active restraint upon the antigen-sensitive cells, which is not present after the cells have been removed, washed and cultured in vivo, or transferred to F_1 recipients. One can postulate that this restraint acts at several stages –

- a block in the early stages of recognition and proliferation

- a block in the late stages of differentiation

- a very late block preventing mature killer cells from attacking the graft.

As the reactivity of unresponsive hosts to other histocompatibility antigens (e.g. C57Bl) is perfectly normal, the block is a highly specific one, and can

act only on those cells triggered directly by donor antigen. The negative HvG reaction strongly suggests that a block in the early stages of recognition may be operative, for further stages in the HvG reaction beyond recognition can be mediated non-specifically.

The evidence for a later block is difficult to assess, particularly because a restraint in the early recognition stages would automatically lead to the absence of differentiated cells. Before antigenic stimulation, there were no cytotoxic cells in the host detectable by the CML test, although this may just reflect a lack of sensitivity of the assay. Also, injection of pre-sensitized cells can break unresponsiveness, although rejection of the graft is rather slow - about 20 days. The survival of the graft on unresponsive mice in the presence of in vivo generated cytotoxic cells may be due to the fact that killer-cells are present, but in insufficient numbers to cause significant damage to the graft. Certainly, in vivo-stimulated unresponsive cells never give as high a cytotoxicity as stimulated normal cells.

So far, almost all attempts to inhibit other assay systems (MLC, GvH, and CML) by either unresponsive cells or serum, have failed. P.J. Kilshaw, L. Brent and M. Pinto, in this laboratory have, however, been able to transfer unresponsiveness by injection of spleen cells into normal, sublethally irradiated or ALS-suppressed syngeneic recipients,[31] in which case unresponsive cells prolong the survival-time of a graft by up to 20 days. A requirement for T-cells has been shown by treating the spleen cells with anti-θ and complement, - a procedure which renders these cells incapable of conferring unresponsiveness. However, it has not yet been proved conclusively that these unresponsive cells when transferred, do not act by

cooperating with recipient B cells to produce a suppressive antibody, so that

suppressor cells, and soluble serum-factors are both equally possible mediators

of suppression at the present time.

If one postulates that suppressor cells are involved, one must explain

the inability of these cells to suppress a GvH reaction in the popliteal lymph

node assay, while at the same time abolishing in situ host-versus-graft activity.

One difference between these two situations is the size of the antigenic dose

to which the responder (and suppressor) cells are exposed. It may well be

that in the GvH system, the small number of unresponsive cells is overwhelmed

by the tremendous stimulus presented by the host tissues, and the suppressor

cells are no longer effective, in contrast to the situation in the unresponsive

host, where the graft and the footpad inoculation both constitute a very small

antigenic challenge. If this is the case, then one might expect some other

tests, such as Elkins' GvH assay under the kidney capsule, to be more easily

suppressed, for here the antigenic stimulus of the host is small.[32]

Experiments along these lines are at present being considered.

It is important to remember that the suppressive mechanism in this

system is highly specific, both in induction and in mode of action. Thus,

pretreatment with donor strain extract is an essential prerequisite for the

induction of unresponsiveness.[33] Also, an experiment performed by P.J. Kilshaw

showed that when the fully unresponsive animal, bearing a perfect A-strain

allograft, is challenged simultaneously with a fresh A-strain graft and a

C57Bl graft, there is no protection of the third-party graft by putative non-

specific factors which may have been brought into action by exposure to a

second challenge of antigen. Instead, this graft is quickly rejected. In fact,

antigen in the form of a primary A-strain graft is always present, and appears to be an essential part of the treatment for induction of unresponsiveness. If the grafting stage on day 0 is omitted, and the graft is applied later, long after the ALS treatment, the mice are fully reactive, and rapid rejection results.

Finally, it must be noted that none of the blocks in the sensitization process are insurmountable. Increasing the antigenic dose may lead to the recognition reaction observed in the GvH and MLC, and certainly produces an increased number of cytotoxic cells in the host. Unresponsiveness can also be broken by treatment of mice at an early stage with cyclophosphamide, or at a later stage with sensitised spleen cells. In both cases, the grafts are speedily rejected. It is evident that in this system we are dealing with a very delicate balance between unresponsiveness and reactivity towards skin allografts.

Chapter III

CHARACTERISATION OF SERUM FACTORS

The passive homing test described in Chapter I showed that the serum of unresponsive mice contains opsonins which can adhere specifically to donor strain lymphoid cells and can divert these cells, when injected into the blood stream of normal mice, from their usual targets, the spleen and lymph nodes, to the liver. The fact that these opsonins bind specifically to donor antigens, and that their action can be modified by C_3 activation, strongly suggests that they are antibodies, or antigen-antibody complexes, but the homing test alone gives no formal proof of this.

To characterise the opsonins more fully, samples of unresponsive serum were fractionated, and the activities of these fractions were measured in the homing test. The techniques of ion exchange chromatography and partition chromatography were employed.

1.   Ion-Exchange Chromatography

   Basic Principle

        Ion-exchange chromatography separates proteins according to their net electrostatic charge. The proteins are bound to a stationary phase (usually a chromatographic column) by electrostatic attraction. Those with a high net charge will be bound more strongly than those with a low charge. The external conditions are then changed, so that the weakly bound proteins no longer adhere to the stationary phase, and can be washed off into the

mobile phase, leaving the strongly bound proteins behind. This is done by reducing the electrostatic binding between column and protein, and can be achieved in two ways, by decreasing either the net charge on the column material, or the charge on the protein.

For serum proteins, the column materials most commonly used for ion-exchange chromatography are DEAE-Sephadex, or DEAE-Cellulose.[1] Both of these are inert polysaccharide support materials, which have been conjugated with positively charged Di-ethyl-amino-ethane (DEAE) groups. The simplest way to reduce the charge on the column material is to introduce chloride ions into the buffer, which compete with the protein for the binding sites on the column and neutralise the charge on the DEAE groups. To reduce the charge on the protein molecules one must introduce positively charged ions into the system, and this is most readily done by lowering the pH - thus increasing the hydrogen ion concentration.

Precipitation of Serum[3]

Before mouse serum is applied to an ion-exchange column, a large proportion of the albumin is removed from the serum by ammonium sulphate precipitation of the globulins. This preliminary step is essential, since it removes 50% of the total unwanted protein contained in the serum, and enables one to use a much smaller column, with consequent increase in resolution. The procedure adopted for precipitation is as follows.[4] 1.2 ml serum, made up to 5 ml with normal saline, is precipitated with 5 ml saturated ammonium sulphate (SAS) in 0.1M TRIS buffer, pH 8, by slow dropwise addition in the cold. The final concentration of ammonium sulphate

is "50% SAS" (2.7 Molar), at which all the globulins are precipitated and the albumin is left in the supernatant. At concentrations greater than 50% SAS, albumin also is precipitated. When the SAS is added dropwise, the protein in solution comes into contact with concentrations of ammonium sulphate greater than 2.7 molar, and in order to prevent local precipitation of albumin, the mixture must be stirred constantly. For the same reason the serum is diluted initially, for the larger the volume of the mixture, the less is the time taken for the SAS to disperse, particularly when the mixture approaches 50% SAS concentration.

It was found that immediately after the SAS has been added, the precipitate is too finely divided to be spun conveniently. Furthermore, a significant proportion of the globulin remains in solution indefinitely if kept at +4 deg C, and can only be encouraged to precipitate on warming to room temperature. Consequently, after addition of SAS, the mixture is transferred to a 10 ml centrifuge tube, and allowed to settle overnight at room temperature before spinning for half an hour at 3000 rpm. The precipitate is then resuspended in 1 ml of 0.01M TRIS-HCl (pH 8) and passed through a G-25 desalting column to equilibrate completely with 0.01M TRIS-HCl. The immunoglobulin is contained in about 10 ml of starting buffer, ready for application onto an ion-exchange column.

Choice of Buffer

A buffer is required which operates effectively at pH greater than 8, because under these conditions all serum proteins bind to DEAE-sephadex. The two buffer systems most commonly used for this are phosphate

Figure 1   Elution profile of 1 ml normal mouse serum from a DEAE sephadex A-25 ion-exchange column, by

application of a continuous salt concentration gradient
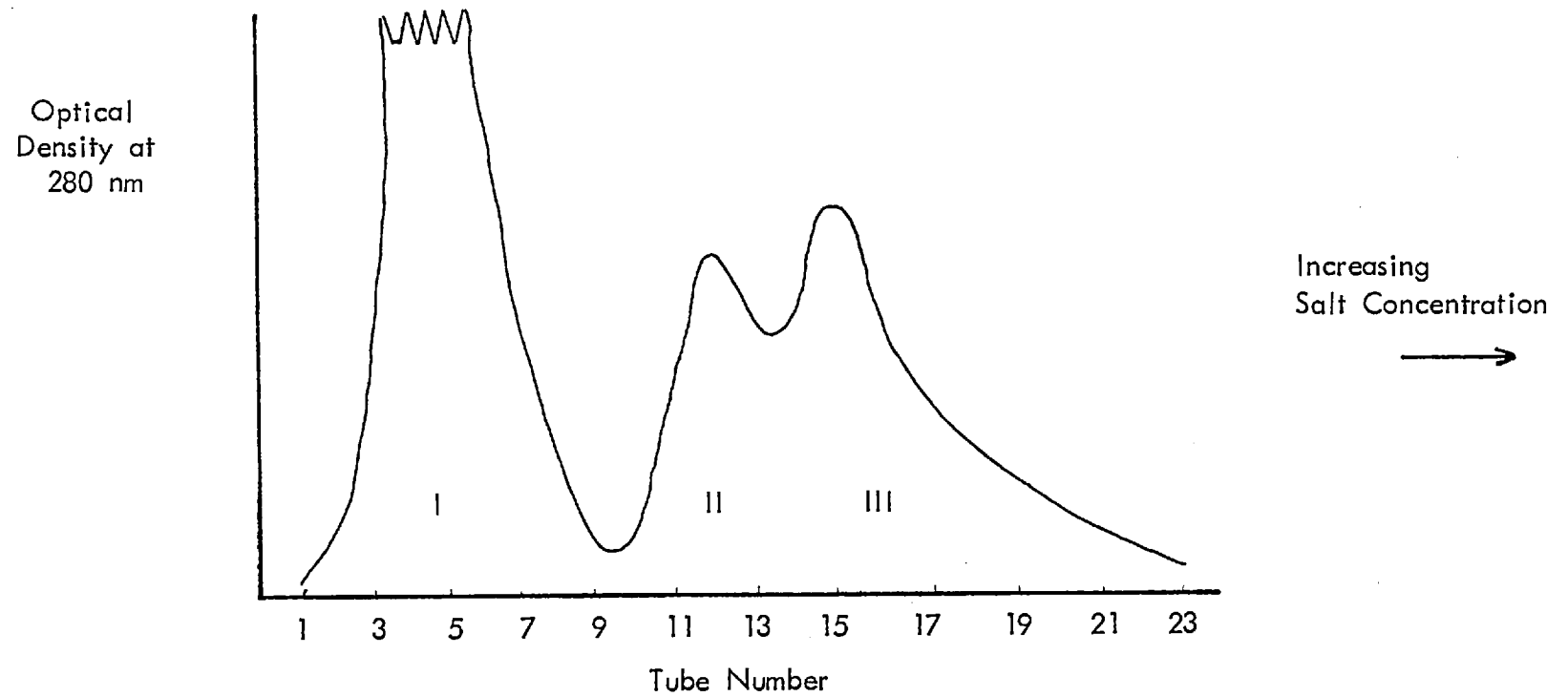
Initial concentration of Cl⁻ in buffer is 0.005 Molar

H_2PO^-/HPO_4^{2-}, and TRIS-HCl. The latter system was chosen, because the TRIS buffer ion does not bind to the column, or compete with chloride ion or protein for DEAE binding sites. This allows greater flexibility in the concentration of buffer ion throughout elution, and permits greater control over the pH at low salt-buffer concentrations than is possible with the $H_2PO_4^-/HPO_4^{2-}$ system. The buffer is prepared by making up a solution of 0.1 molar TRIS in distilled water, and adding concentrated hydrochloric acid until the pH descends from about 11.5 to 8.0. Approximately 4 ml of concentrated (36%) hydrochloric acid is required per litre of TRIS, which results in a concentration of 0.05M chloride ion. This can be diluted ten-fold in distilled water to give the starting buffer, referred to as 0.01M TRIS-HCl. The actual concentration of Cl^- ion is very close to 0.005 molar.

Gradient Elution

Proteins are bound to the column at very low salt concentration, and are removed by increasing the amount of chloride ion in the eluant buffer.[5,6] Different proteins are eluted at different times according to the net electrostatic charge of each protein, the least charged (i.e. the most weakly bound) coming off first at low chloride ion concentration. Figure 1 shows an elution profile for normal mouse serum, precipitated as described. The concentration of salt is increased from 0.005M to 1M, and the final concentration of TRIS is 0.1M. The column material is DEAE-sephadex, with a bed volume of 15 ml. Under these conditions, practically all the binding sites on the column are filled by protein initially. The relative protein concentration in the eluant is measured by an on-line Uvicord Spectrophotometer

Figure II   Elution profile of normal mouse serum from a DEAE sephadex A-25 ion-exchange column by application
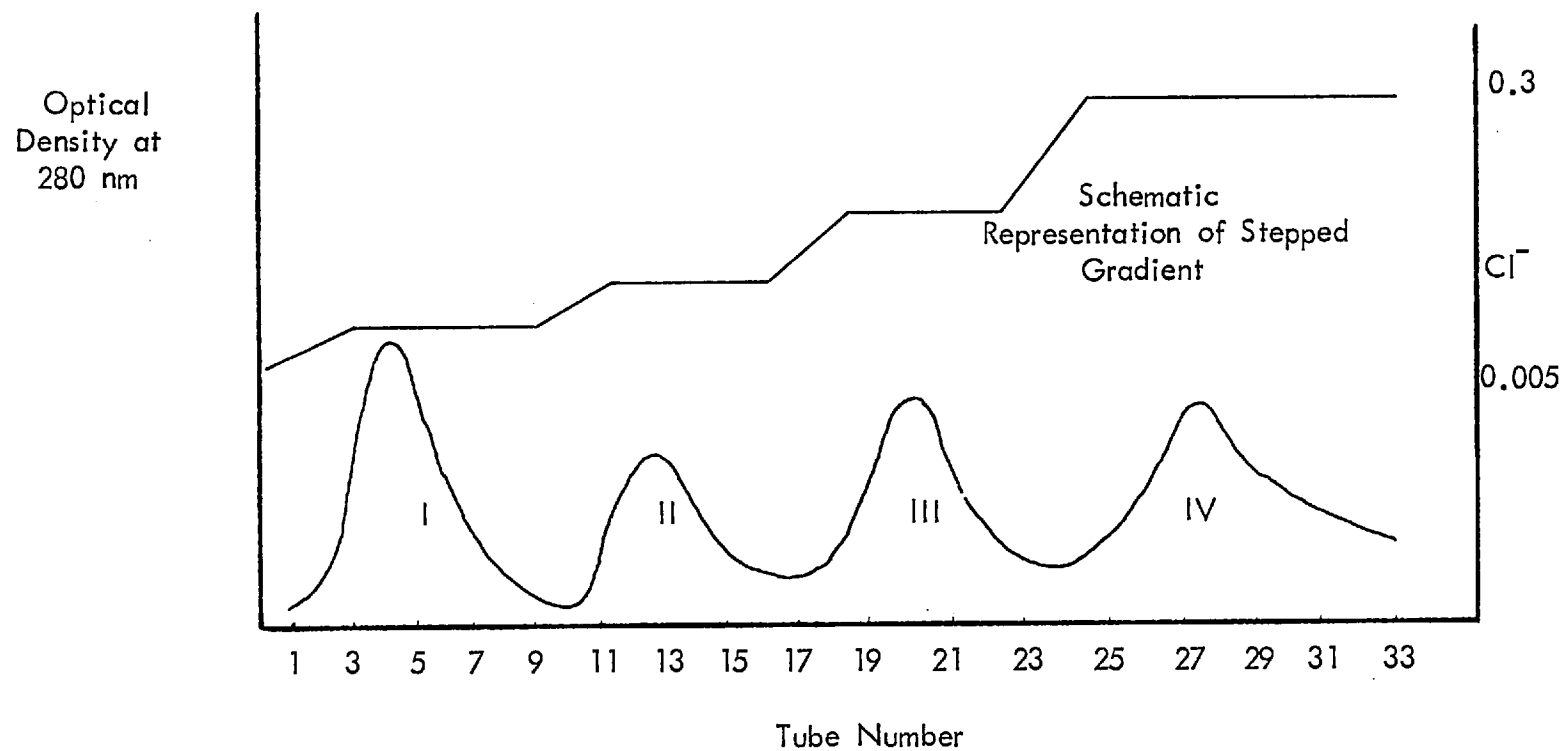
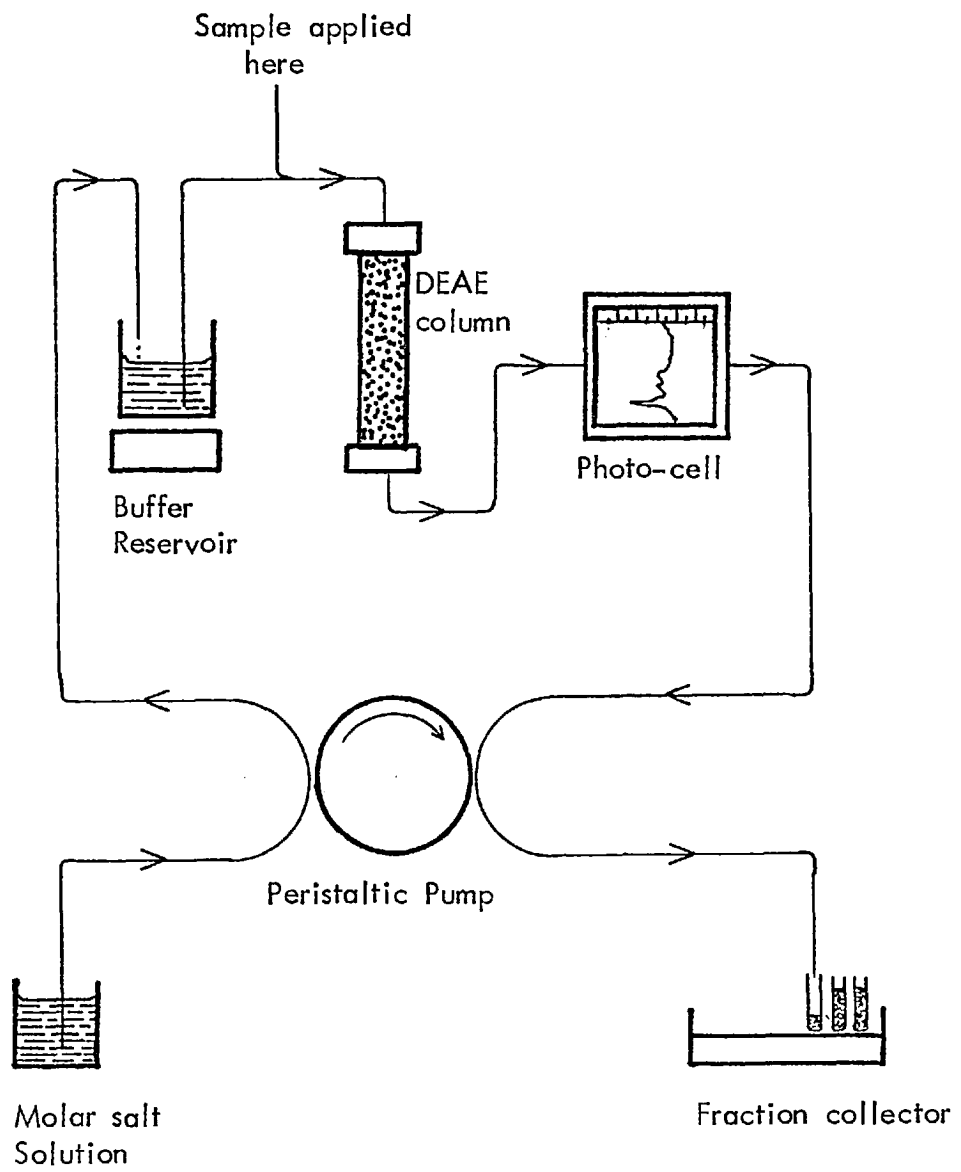of a stepped salt concentration gradient

Diagram I   Lay-out of Apparatus for Ion-exchange Fractionation

cell (measuring absorption at 280 nm) before collection by a LKB 7000 Ultrorac fraction collector. The speed of buffer flow (15 ml/hr) was maintained constant by the use of a peristaltic pump (see Diagram I).

To obtain greater resolution, the gradient can be halted as soon as one peak starts eluting, and the column washed out with buffer of low salt concentration. This is done by stopping the addition of salt solution to the buffer reservoir as soon as the peak is observed. The increased separation between different peaks is demonstrated in Figure II. The interruptions in the gradient are indicated by the dotted line, which represents schematically the concentration of chloride ion throughout the elution. Analysis using heterologous antibody class-specific antisera in an Ouchterlony plate (not shown here) revealed the following constituents in the fractions of normal mouse serum; fraction 1 - IgG, fraction 2 - IgM, fraction 3 - IgA. The fourth fraction is presumably albumin. Traces of IgG1 are also present in fractions 2 and 3. The antisera were kindly donated by Dr Frank Hay of the Middlesex Hospital Medical School.

The efficacy of the separation depends very much on there being as little distance as possible between the spectrophotometer cell and the buffer reservoir (Diagram 1) for the salt concentration in the reservoir is always greater than that necessary to elute the protein peak observed on the spectrophotometer. Thus it is advantageous for the column to be as small as possible. For this reason, DEAE-sephadex A-25 was chosen as the column material: because of its high capacity for protein, only a small amount of column material is required.

Sample Application

It was found that the binding of protein to the column is a time-dependent process. For the protein in the sample to be fully retained, even at the lowest concentration of Cl^- ion, it is necessary to apply the sample very slowly over a long period of time. At high application speeds, the more weakly binding proteins pass straight through the column without sticking. Equilibration of proteins within the column is probably related to the accessibility of binding sites on the sephadex. In cases where the DEAE groups are not in excess, a certain time must be allowed for the proteins to rearrange themselves, so that the highly charged proteins are not covering all the sites, and even the most weakly charged proteins can find enough DEAE groups to bind firmly.

The most successful procedure ensuring complete retention was to make the sample up to 10 ml (i.e. diluted ten times with respect to normal serum) and to run it into the column at a rate of 5 ml per hour. The sample is then left on the column overnight, at room temperature, before washing with buffer and eluting with a chloride ion concentration gradient.

Homing Activity of Fractions

After the different fractions are eluted from the column, they are each equilibrated overnight with physiological buffer (PC) and then concentrated (from about 10 ml to 1 ml) on a Diaflo concentrator. The pore size of the filter is such that it will retain all proteins of molecular weight greater than 10,000. Each of the fractions of volume 1 ml is then incubated with

Table I   Homing Test:

Incubation of strain A cells with same amount of hyperimmune

serum in different incubation volumes

Serum	Dilution	Incubation Volume (ml)	Homing Index
HYP	$^1/1000$	0.4	6.2
HYP	$^1/5000$	2.0	5.8
MED	–	0.4	1.4

HYP: hyperimmune serum

MED: medium only (PC)

Figure III   Elution profile of hyperimmune serum from a DEAE-sephadex ion exchange column by application of salt gradient.   Homing activities of individual fractions.
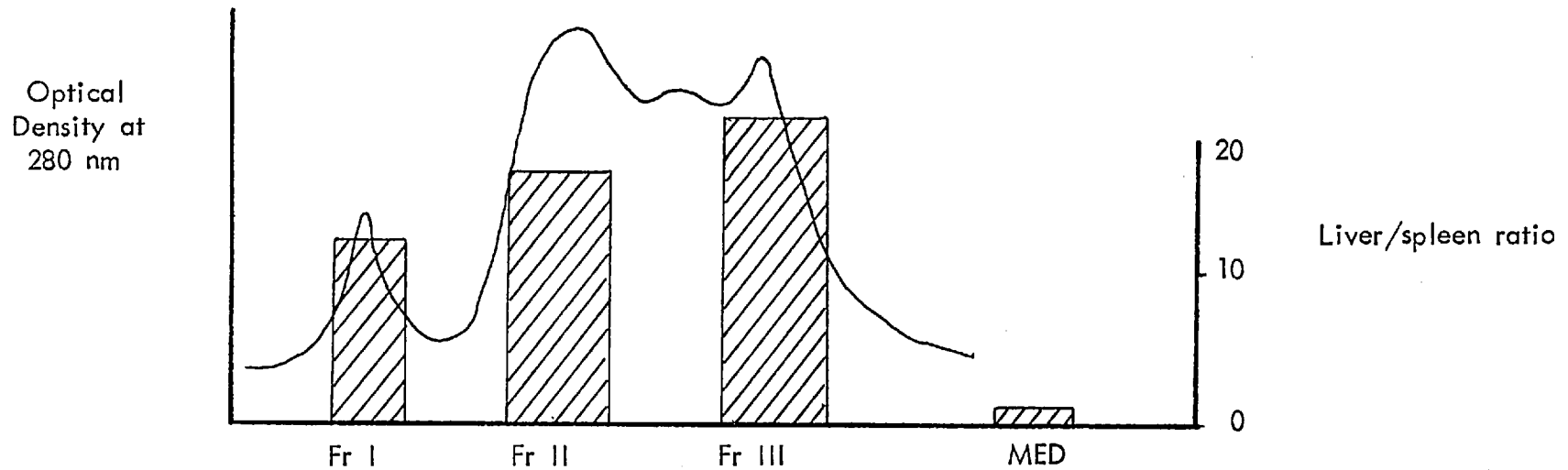
The histograms represent the homing activity of labelled strain-A lymph node cells after incubation with the fraction corresponding to the protein concentration peak superimposed.   MED is a negative control in which cells were incubated with medium alone.   The height of each column represents the magnitude of the homing index, with reference to the scale on the extreme right.

Figure IV   Elution profile of "unresponsive" serum from a DEAE sephadex ion exchange column by application of a

stepped salt concentration gradient: Homing activities of individual fractions: Experiment I

Optical
Density at
280 nm

Fr I   Fr II   Fr III   Fr IV   MED

Liver/spleen ratio
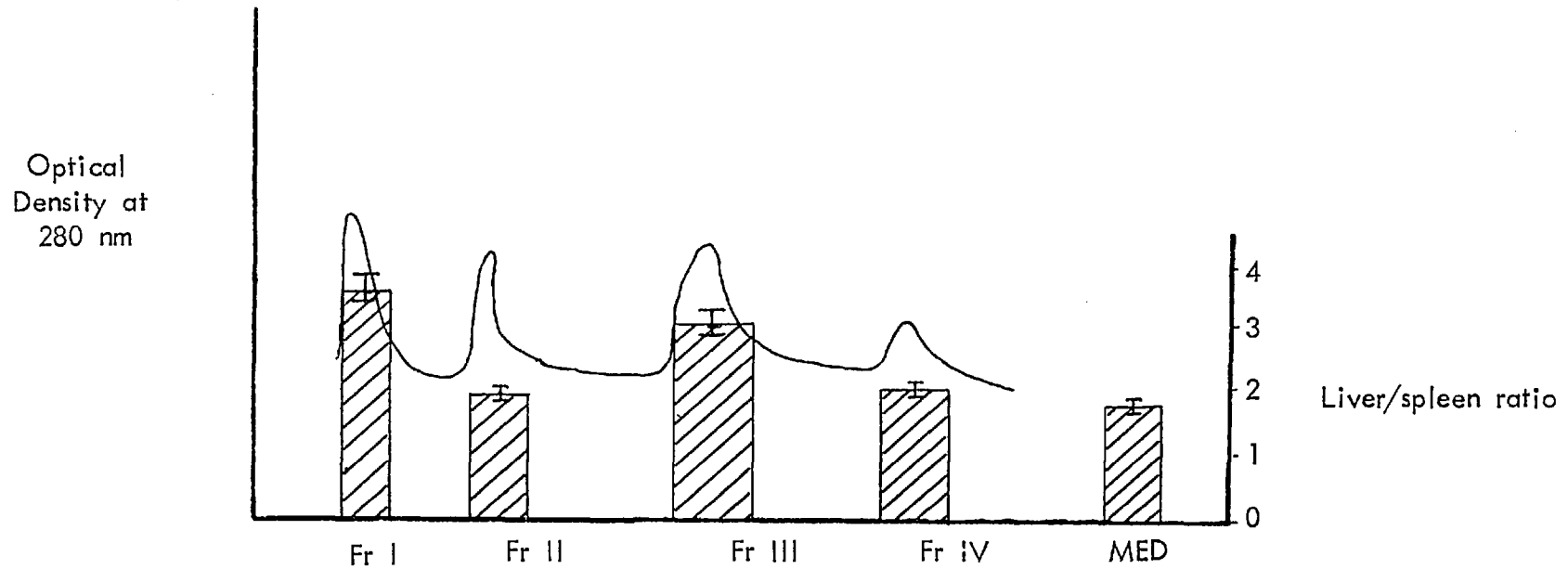
The histograms represent the homing activity of labelled strain A lymph node cells after incubation with the fraction corresponding to the protein concentration peak superimposed.   MED is a negative control in which cells were incubated with medium alone.   The height of each column represents the magnitude of the homing index with reference to the scale on the extreme right.

Table II   Homing Test

Absence of homing activity in the supernatant obtained after

precipitation of "unresponsive" serum with saturated ammonium

sulphate

Serum	Homing Index
HYP	8.6
URS (untreated)	6.4
* SAS precipitate	6.2
* SAS supernatant	3.0
MED	3.2

* Obtained by precipitation of serum from unresponsive mice with 50% saturated ammonium sulphate in 0.1M TRIS buffer – pH 8.0

Figure V   Elution profile of "unresponsive" serum from a DEAE sephadex ion-exchange column by application of a

stepped salt concentration gradient.   Homing activities of individual fractions: Experiment II
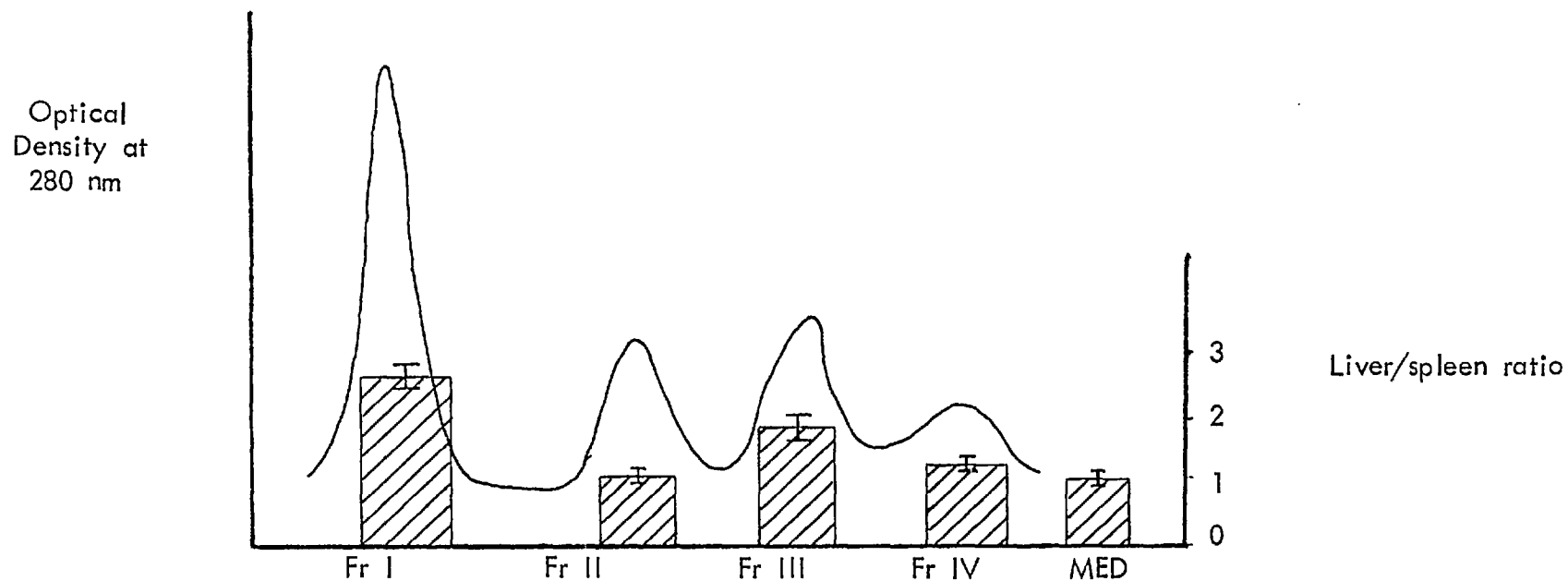
The histograms represent the homing activity of labelled strain A lymph node cells after incubation with the fraction corresponding to the protein concentration peak superimposed. MED is a negative control in which cells were incubated with medium alone. The height of each column represents the magnitude of the homing index, with reference to the scale on the extreme right.

Plate I   Ouchterlony Plate
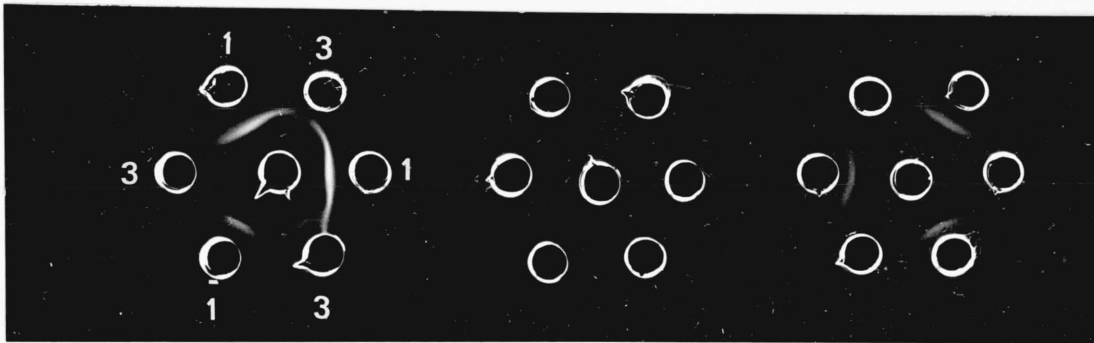
Identity of fractions obtained from ion-exchange separation

of "unresponsive" serum

Class-specific antibody in central well

Anti-IgG   Anti-IgM   Anti-IgA

Wells marked 1 contain samples of fraction I from the separation shown in

Fig. V, in three different concentrations

Wells marked 3 contain samples of fraction III from the separation shown in

Fig. V, in three different concentrations

4 million ^{51}Cr-labelled A-strain lymph node cells, and the cells are injected into groups of five normal CBAs as in the homing test described in Chapter 1. In a preliminary experiment it was found that increasing the incubation volume from 0.4 ml to 1 ml made very little difference to the homing index of a sample of diluted hyperimmune serum (Table I). Figure III shows an elution curve for hyperimmune serum, the fractions of which were subsequently tested for homing activity. The height of the shaded bars represents the homing index for each of the fractions. It can be seen that all three fractions contain substantial amounts of opsonins, compared with a medium control (on the extreme right).

Fractionation of Unresponsive Serum

The results of homing tests performed on fractions of unresponsive serum are summarised in Figure IV. Table II shows that the albumin-containing supernatant from the SAS precipitation has no homing activity. In the two experiments shown in Figures IV and V, there appears to be significant activity in fractions I and III. These two fractions were identified by means of class-specific antisera in an Ouchterlony plate (see Plate I) as IgG and IgA respectively. It was felt that the Ion-exchange technique was not sufficiently developed to warrant attempts to separate IgG into its subclasses, although Dr Frank Hay[7] has reported some recent work in which mouse IgG_1 and IgG_2 immunoglobulins were separated using a method very similar to the one described here.

2. Sephadex G-200 Fractionation[8,5]

Separation of serum components according to their molecular size was also undertaken, in order to provide further evidence of the antibody nature of the serum opsonins in unresponsive serum. The column used had a bed volume of 600 cc and a length of 80 cm. A sample of approximately 10 ml could be applied to the column without line broadening, provided the sample was of low enough viscosity. The flow rate was 1 to 10 ml per hour, and the column material was sephadex G-200, which retains proteins of molecular weight 200,000 and under (in particular, IgG) and lets higher molecular weight proteins (IgM, and IgA) flow straight through with the eluant buffer. The time taken for the separation on the column is at least 24 hours, and the initial precipitation and final concentration steps are both time consuming so that the total separation takes several days. To shorten the time required, the buffer chosen was a physiological buffer, PC, so that there was no need to equilibrate the different fractions with PC overnight, after separation, before using them in the homing test. To prevent bacterial growth, gentamycin 10 IU/ml, ampicillin 125 μg/ml, and cloxacillin 125 μg/ml, were added to the buffer. The precipitation, final concentration steps and the homing were carried out exactly as described before. Figure VI shows an elution curve for unresponsive serum separated on G-200 sephadex, the fractions of which were subsequently tested for homing activity. Table III shows the homing indices for each fraction. The identity of each of the fractions is shown by the Ouchterlony plate (see Plate II). It can be seen that fraction I, containing only IgM and IgA has no activity. Fraction III, containing 7S IgG   , has substantial activity, while the

Figure VI   Elution profile of "unresponsive" serum from a G-200 sephadex column by application of a physiological

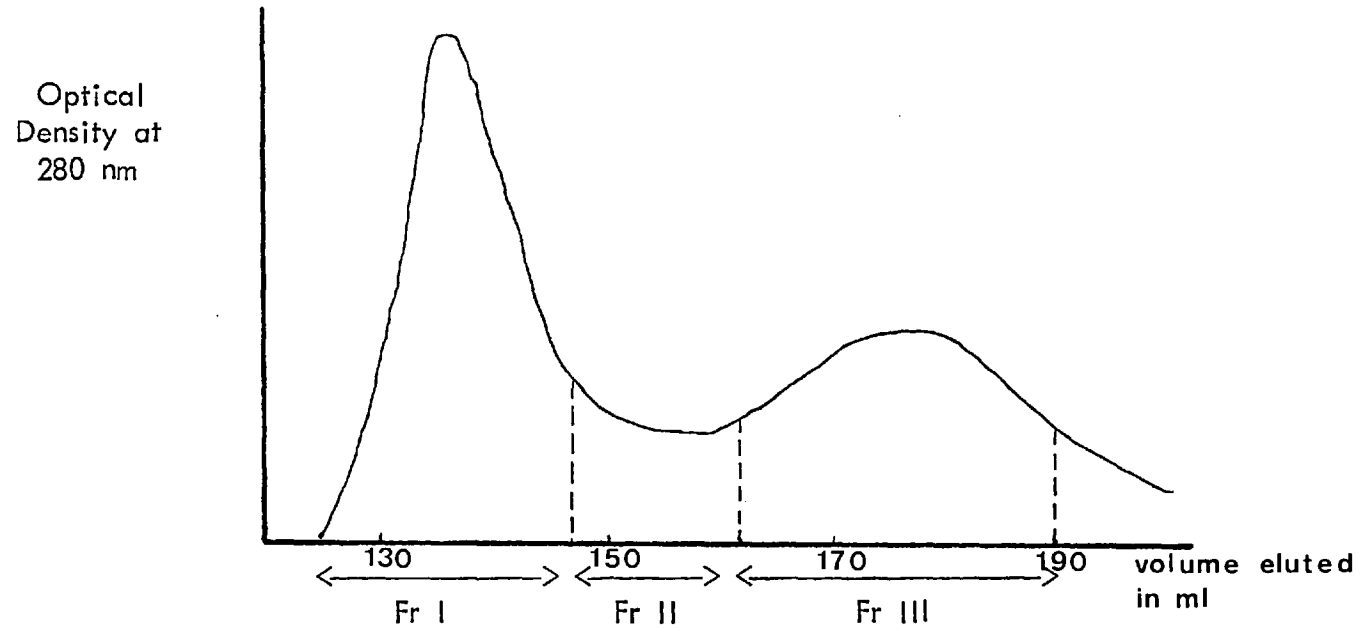buffer of constant salt concentration

Table III   Homing indexes of labelled strain A cells incubated with

fractions of "unresponsive" serum obtained from the G-200

sephadex separation illustrated in Figure VI

Fraction	Homing Index
MED*	1.7
Fr I	1.3
Fr II	2.4
Fr III	3.5

* MED is a control in which cells were incubated with medium which had also passed through the column.

In this particular experiment, the albumin fraction had been removed by ammonium sulphate precipitation to such an extent that the third peak which normally appears upon G200 Sephadex fractionation (due to albumin) was scarcely discernable, and consequently was not included on the elution profile in Figure VI. The marker proteins used to calibrate the column were those intrinsic in normal and experimental sera. The void volume was 120 ml, and the albumin peak (identified by electrophoretic mobility) came when a total of 250ml was eluted from the column after application of a sample of normal serum.

Plate II   Ouchterlony Plate
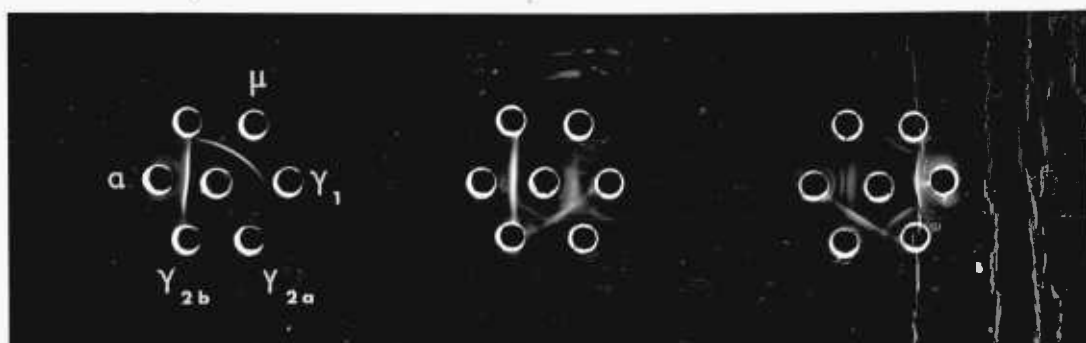
Identity of fractions obtained from G-200 separation of

"unresponsive" serum

Fractions in Central Wells

Fr I   Fr II   Fr III

Symbols attached to the peripheral wells indicate the class-specificity of
heterologous anti-Ig antibody in each well

activity of fraction II, which has small amounts of all classes, is considerably less. The experiment has been repeated twice, with the same result in each case.

Assay for Complexes

It was hoped that the fractionation techniques described above might answer the question of whether complexes play a part in the opsonization of donor strain lymphoid cells. Until this is known one cannot be sure that the serum factor associated with IgG in the ion-exchange separation does not in fact belong to some other immunoglobulin class, but which, as a result of binding to an antigen fragment, has had its electrical properties modified. On the other hand the fact that no homing activity has been found in the excluded volume of the Sephadex G-200 separation implies that neither free IgM or IgA, nor complexes associated with these antibody classes, can be responsible for homing activity, because such complexes would also be associated with this first peak. However, the homing activity observed in the third fraction in the ion-exchange separation (associated with IgA) could be due to IgG antibodies bound to antigen fragments. One might expect such complexes to be found between the 19S IgM and 7S IgG peaks in the Sephadex fractionation (i.e. fraction II in the Figure V), and indeed this fraction does display a considerable degree of homing activity. Unfortunately it is impossible to determine whether this activity is due to complexes, or to weakly bound aggregates of IgG formed upon storage, or just to the poor resolution of the IgG peak, which overlaps slightly with the peak I.

A different approach therefore was adopted to test for the presence of complexes in the serum of unresponsive mice. A technique developed by Onyewotu and Holborow[9] for assaying the sera of patients with systemic lupus erythematosis was adapted for the mouse system. The method relies on the fact that antigen-antibody complexes, and aggregated antibodies, are bound non-specifically to the surface of macrophages via F_c receptors. This binding can be observed by measuring the uptake of ^{125}I by macrophages after incubation with heat-aggregated ^{125}I-normal immunoglobulin, and it can be inhibited by addition of cold antigen-antibody complexes to the incubation mixture, which compete for the same (or closely related) receptor sites on the macrophage surface.

The experimental procedure was as follows. Five normal CBA females were injected i.p. with 0.3 ml Incomplete Freund's Adjuvant each. Three days later the animals were killed and the peritoneal cavities were washed out with 5 ml phosphate-buffered PC. The yield of peritoneal cells was about 20×10^6 per animal, of which more than 75% were macrophages. The cells were washed twice in PC, and the suspension allowed to stand for ten minutes to enable cell clumps to settle out and be removed. This settling procedure was performed twice, to give a pure single cell suspension. The cells were then made up to a concentration of 1.5×10^6 /ml in PC with 10% normal mouse serum added, and incubated for 45 minutes at 37 deg C. This preincubation with normal mouse serum was performed in order to prevent binding of aggregated immunoglobulin to the macrophages. 0.5 ml aliquots of the suspension were then transferred into 2 ml plastic capped tubes, and up to 50 μl aggregated ^{125}I-immunoglobulin was added, together with a similar

Figure VII   Uptake of Iodine-125 labelled normal mouse immunoglobulin aggregate by 7.5×10^5 peritoneal

macrophages: Effect of dose of labelled aggregate during incubation
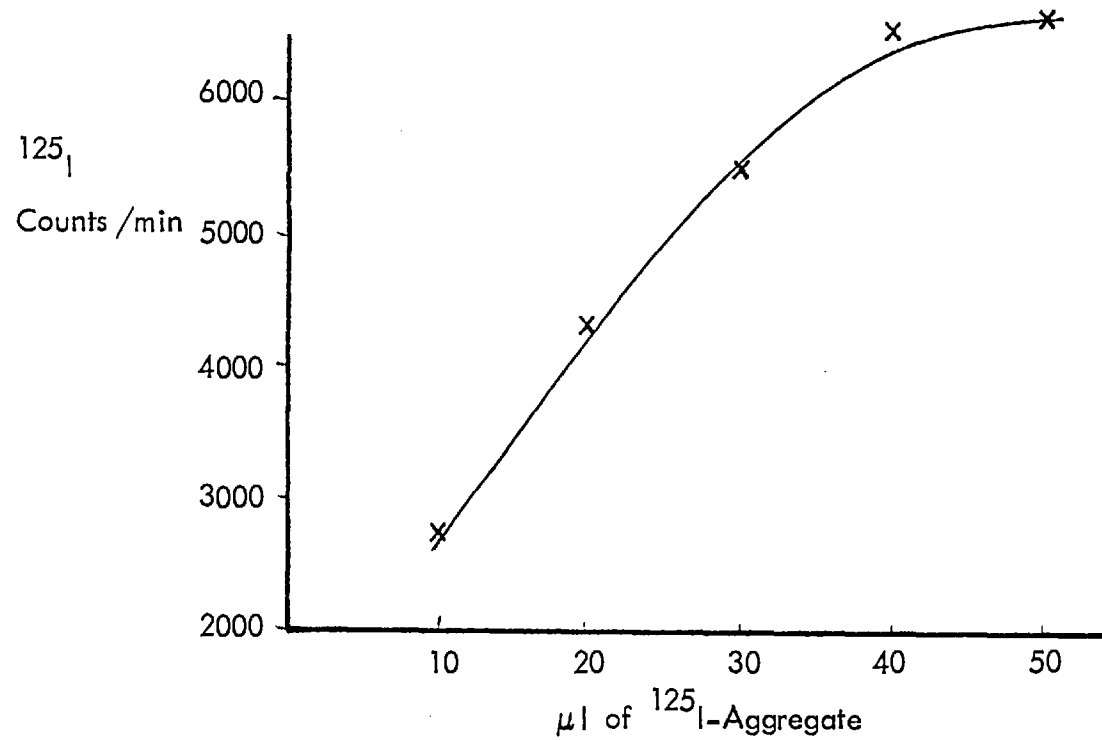
Macrophages were from 3 month old CBA males
Each point is the mean of three results
The incubation time was $1^3/4$ hours for all doses

Table IV   Uptake of Iodine-125 labelled normal immunoglobulin aggregate

by 7.5×10^5 peritoneal macrophages: Inhibition by cold

aggregated mouse serum

	Labelled Material	Cold Inhibitor	Uptake of ^{125}I Counts/min
1	10 μl ^{125}I-AGG	10 μl NMS	2,160
2	10 μl ^{125}I-Agg	10 μl Agg MS	1,100
3	10 μl ^{125}I-NIG	10 μl NMS	1,860
4	10 μl ^{125}I-NIG	10 μl Agg MS	1,660

^{125}I-NIG: labelled normal immunoglobulin

^{125}I-AGG: labelled normal immunoglobulin aggregate

NMS: normal mouse serum

Agg MS: aggregated mouse serum

Table V   Uptake of Iodine-125 labelled normal immunoglobulin

aggregate by 7.5×10^5 peritoneal macrophages:

Effect of "unresponsive" serum

Labelled Material	Cold Inhibitor	Uptake of ^{125}I Counts/min
50 μl ^{125}I-AGG	–	5230
50 μl ^{125}I-AGG	10 μl Agg MS	3200
50 μl ^{125}I-AGG	10 μl NMS	4350
50 μl ^{125}I-AGG	10 μl URS	4070

^{125}I-AGG: labelled normal immunoglobulin

Agg MS: aggregated mouse serum

NMS: normal mouse serum

URS: serum from unresponsive mice

quantity of the labelled test substance. The mixture was incubated for one hour, then the cells were spun down and washed in situ six times to remove any label not bound to the cells. The tubes were then counted in a Packard Scintillation counter. All measurements were made in triplicate.

The labelled material was produced by separating the IgG fraction from normal or unresponsive mouse serum by DEAE ion-exchange chromatography (after ammonium sulphate precipitation), labelling the IgG with ^{125}I by the Chloramine T method,[10] and then removing unbound ^{125}I by passing the mixture through a G-25 sephadex column. The column was presaturated with BSA carrier protein (1 mg/ml) in order to minimise losses of immunoglobulin. The material was then aggregated by heating at 63 deg C for $\frac{1}{4}$ hour. After spinning at 1000 g, the supernatant was removed, and used neat in the test. The concentration of IgG was about 15 μg/ml. As positive and negative controls for inhibition, aliquots of aggregated and unaggregated normal mouse serum were used.

The uptake of label in the absence of inhibitory material was investigated in a preliminary experiment (Fig. VII). The quantity of material bound appears to reach a plateau in the presence of 50 μl of aggregate, when presumably all the receptor sites on the macrophage surface have been filled. Table IV shows the results of an experiment in which the uptake of label was inhibited by the addition of cold aggregated normal mouse serum (compare groups 1 and 2). The ability of aggregated mouse serum to inhibit uptake of label has been confirmed in two other experiments. It also shows that aggregated mouse serum does not compete with normal unaggregated immunoglobulin for binding sites on macrophages (groups 3 and 4).

A similar experiment was performed with unresponsive serum and the results are given in Table V. The quantity of labelled material in the incubation mixture was increased to 50 μl to achieve saturation of the cell surface. Under these conditions, cold aggregate inhibited compared with the normal serum control but unresponsive serum did not. The experiment was repeated, using 50 μl of test material, but the result was the same.

This assay is claimed to be one of the most sensitive tests for complexes developed, capable of detecting as little as 25 ng of material in 10 μl of human serum. In the experiments reported here, however, the lower limits of sensitivity were not tested, because the positive controls contained aggregated material in high concentrations, comparable with that of total protein in normal serum. The negative result in this test does not therefore exclude the possibility that complexes are present in the serum in very small quantities.

Discussion

The serum factor active in the homing test is associated with the IgG fractions in both ion-exchange and sephadex G-200 separation techniques. An in vitro test performed by Dr P.J. Kilshaw confirms this finding, in which the binding of unresponsive serum to ^{51}Cr-labelled A-strain red cells after incubation was measured by uptake of ^{125}I on incubation with iodinated sheep anti mouse-immunoglobulin antisera. The antisera were class-specific, and only those directed towards IgG_1 and IgG_{2a} bound to the red cells. An analogous experiment is possible with the homing system, in which antibody-mediated homing can be enhanced by incubating strain A cells first with

unresponsive serum (as usual) and then, after washing, with heterologous

anti mouse-immunoglobulin sera. Binding of the anti-Ig sera to the cell

membrane will occur via the anti H-2 antibodies in the unresponsive serum,

and the increased number of F_c moieties on the cell surface should result in

a greater proportion of these cells being found in the liver. A preliminary

experiment, however, using thymocytes as lymphoid cells, was unsuccessful

because these cells have a very high non-specific uptake of normal serum

proteins, which leads to a high background of anti-Ig. This binding of serum

proteins (in particular immunoglobulin) may account for the increased tendency

of normal thymocytes to home to the liver, as they will have large numbers of

F_c chains on their surface, and this encourages uptake by macrophages and

Küpffer cells. Similar experiments are in progress using mouse red blood cells,

the distribution of which is also affected by antibody covering their surface.

There are several mechanisms by which serum factors could mediate

unresponsiveness. Homing tests demonstrate very clearly the way in which

antibody is able to clear antigen from the circulation, and such a procedure

may effectively remove the stimulus required to turn antigen-sensitive

precursors into mature killer cells. Whether this mechanism is effective in

the inhibition of the host-versus-graft reaction is doubtful, however, for other

experiments (see Chapter 1) showed that there is no significant reduction of

antigen in the lymph nodes of unresponsive mice after injection of F_1 cells

into the footpad.

A second possibility is that the antigenic sites on the graft are masked

by antibody, so that even in the presence of mature effector cells no cell-

mediated attack on the graft can be mounted. Certainly, even when cytotoxic

cells are stimulated in unresponsive hosts by injection of F_1 antigen, no damage to the graft is observed, although it is rejected upon adoptive transfer of sensitized spleen cells. Dr P.J. Kilshaw attempted to detect antibodies on the vascular endothelium of the graft by incubating sections with iodinated anti-immunoglobulin but he found no difference after autoradiography between the amount of label adhering to CBA and A-strain grafts from the same unresponsive animal.

Serum factors could alternatively act by blocking the receptor sites on the surface of antigen-sensitive cells. The factor would need to contain an antigenic moiety which will bind to the cells without triggering it to proliferate. Association of these antigenic determinants with antibody may help them to remain on the surface of the cell, and so prevent the cells from coming into contact with more strongly immunogenic stimuli. Furthermore, cells coated with such antigen-antibody complexes would be predisposed to home to the liver and be destroyed thereafter, although GvH and MLC tests suggest that the antigen-sensitive clone of cells in unresponsive mice is not depleted.

Alternatively, blocking of the receptor sites could be achieved by anti receptor-site antibody (anti R-S). McKearn and co-workers[11] have claimed that such antibodies are responsible for the suppression of a graft-versus-host disease in normal rats, and Binz, Lindemann and Wigzell have demonstrated inhibition of the popliteal node GvH reaction by anti R-S.[12] No data for the host-versus-graft system are available. The latter would certainly be more difficult to inhibit, because the pool of antigen-sensitive cells to be suppressed is much larger. In most work involving anti R-S,[13,14,15] the

antibodies have been raised by the action of immunocompetent hybrid cells either against parental lymphocytes, or against parental antibodies. It is assumed that there is a requirement for a population of cells which is allogeneic with respect to the antibody forming cells so that the receptor sites are recognised as being foreign. To explain the presence of anti R-S antibodies in unresponsive mice, one would have to postulate a completely different mechanism of induction, for at no time during the treatment inducing unresponsiveness are allogeneic lymphoic cells introduced into the host.

No experiments have been performed which either confirm or deny the presence of anti R-S antibodies in unresponsive animals. The homing test is unlikely to detect them, because it observes the effect of antibody on a large population of cells in which the antigenic determinants are spread homogeneously throughout, whereas anti-idiotype antibodies are expected to bind to only a very small proportion of the total cell population. On the assumption that the receptor sites of lymphocytes are identical with those of antibodies directed towards the same antigens, attempts to inhibit binding of hyperimmune serum with donor strain antigen, by incubation with unresponsive serum, are now being carried out by Dr P.J. Kilshaw. Unfortunately, these experiments may not distinguish between anti R-S antibodies, and antigen-antibody complexes. Also, if anti R-S antibodies are present, one would certainly expect complexes between anti R-S and the anti donor strain antibodies which were detected by the homing test.

The results given in this chapter demonstrate that the homing activity of serum from unresponsive mice is associated with 7S immunoglobulin. However, attempts to determine whether this antibody is present either in the free form, or aggregated, or bound to antigen, have been unsuccessful.

SUMMARY AND CONCLUSION

Unresponsiveness towards H-2 incompatible skin allografts can be induced in adult CBA mice by pre-treatment with a liver antigen extract, Bordetella pertussis vaccine, and a limited course of ALS injections shortly after application of the graft. As a result of this treatment, 30 - 50% of the recipients retain their grafts for longer than 100 days, even after challenge with other donor antigens such as spleen cells. The phenomenon is strain specific. Although the humoral response is very weak, no haemagglutinating or cytotoxic antibodies being present at any stage, cells from these mice have virtually normal reactivity towards donor strain antigens in the MLC, CML and splenomegaly assays.

This state of unresponsiveness to skin allografts was investigated using the popliteal lymph node assay, which can be a measure of both graft-versus-host and host-versus-graft reactions. Preliminary experiments were carried out to determine the optimal cell dose, the effect of washing with foetal calf serum, and the effect of dead cells in the donor cell inoculum upon the index of reactivity in the test. A modification was introduced in which the response was measured by uptake of ^{125}IUdR in the lymph nodes.

Cells from unresponsive mice were shown to have normal reactivity in the graft-versus-host assay, in which normal $(A \times CBA)F_1$ were used as hosts, confirming the results of MLC and splenomegaly experiments. However, the host-versus-graft test showed that the response to donor strain antigens was markedly reduced when unresponsive animals were the hosts.

It is reasonable to postulate that the same mechanism is responsible for

the inhibition of HvG reactivity, and the prevention of skin graft rejection.

Further work was directed towards inhibiting a normal reaction in the popliteal lymph node by passive administration of either cells or serum from unresponsive mice. Two graft-versus-host and two host-versus-graft experiments showed a small but consistent depression of reactivity upon injection of unresponsive serum, but results obtained for adoptive transfer of cells in two host-versus-graft experiments were inconclusive. The conclusions to be drawn from the work presented above are as follows:

1)   There exists in the unresponsive animal a clone of cells capable of reacting towards donor strain antigens.

2)   These cells are prevented from responding in the unresponsive host by an active restraint mechanism that almost certainly involves a block in the early recognition stage of the development of the immune response.

3)   Unresponsiveness, as observed in the popliteal lymph node test, can be partially conferred by the injection of serum from unresponsive mice into normal recipients.

This is in contrast with the finding that "unresponsive" serum does not prolong skin allograft survival in ALS treated recipients, and with totally negative attempts to inhibit MLC and CML reactions with such serum.[1] The failure of the in vitro tests to demonstrate suppressive effects of unresponsive serum may be due to the inadequacy of culture conditions, or the insufficiency of serum used in culture (20% was the highest concentration used in MLC). In the case of the in vivo system, using ALS treated mice, the serum was injected one day after application of the graft, and it might be that a different time interval would produce different results.

The nature of the suppressive serum factor, however, has not been established, nor has the type of cell producing it, nor even the type of cell upon which it acts. Indirect evidence suggests that it is highly antigen specific, both in its induction and mode of action, for it does not suppress a reaction in the unresponsive host towards third-party antigens presented simultaneously with a donor strain challenge. Conventional antibodies (possibly complexed with antigen) are therefore strong candidates for the mediators of suppression, although this does not exclude the possibility of additional T-cell mediated mechanisms.

Further tests on unresponsive serum, using a modification of Lance's homing technique, showed that antibody is indeed present. This technique observes the way in which the fate of chromium-labelled donor strain lymphoid cells, 24 hours after injection into CBA recipients, is altered by the presence of anti donor strain antibodies coating the surface of the labelled cells. Antibodies in the unresponsive serum were detected by incubating the ^{51}Cr-labelled normal A-strain cells with the serum just before injection into normal CBAs, and observing the ratios of radioactivity recovered in the liver and spleen. The homing index of unresponsive serum was the same as that for hyperimmune serum diluted 1/1000. The activity of the unresponsive serum left over after one exposure to A-strain cells in a homing test was measured by incubating the same serum with a second batch of labelled cells and injection of these into a new batch of CBA recipients. This experiment showed that exposure to donor strain cells removed very little of the active factor from the unresponsive serum, a result which could be attributed either to low avidity, or restricted specificity of the antibody. However, the activity could not be

detected when the serum was diluted ten-fold.

Depletion of C_3 in the serum of recipient mice by CVF treatment showed that complement was a factor in the antibody-mediated homing of lymph node cells, and that the homing index of unresponsive serum too, was complement-dependent.

Separation of unresponsive serum on chromatographic columns was followed by an assay of the activity of each of the fractions in the homing test. Activity was found to be associated with the IgG (and possibly IgA) peak on an ion-exchange column, and with a 7S peak of G-200 separation. A technique involving uptake of aggregated material on macrophages failed to detect the presence of antigen-antibody complexes, whether of donor strain origin or otherwise. The conclusions are summarised below:

1) The serum contains IgG which binds specifically to donor strain lymphoid cells.

2) A proportion of the antibody is complement-binding.

3) The antibody is in extremely low concentration.

4) The antibody cannot cover the surface of donor strain lymphoid cells completely, either because it is of low avidity or because it is directed towards only a small proportion of the total surface alloantigenic determinants.

5) It is unlikely that the antibody is highly aggregated, or that it exists in the form of complexes to any great extent.

Several difficulties were encountered during the course of the project which limited the choice of experiments that could be performed in the allotted time. A perennial problem was the supply of unresponsive mice.

Wherever possible, work was carried out on mice which had carried healthy donor skin grafts for at least 100 days, because it was considered that these mice were in a stable state of unresponsiveness and that there was little chance of their being close to a rejection crisis, which could make results difficult to interpret. Previously obtained data from this laboratory had indicated that after 100 days or thereabouts the remaining grafts showing complete survival are retained indefinitely. Unfortunately, only about 30% of the treated mice still carry healthy grafts at this time, and this was an important limiting factor. Because of an acute shortage of inbred mice at St. Mary's during the last two years there were inevitably many lines of investigation which could not be followed up.

A second problem concerns the immunological reactivity of the unresponsive mice. They received their grafts when they were about three months old. By the time they were used in analytical experiments they were at least six months old, having kept their grafts for a minimum of three months. Consequently, much experimental work was carried out in mice whose overall immunological reactivity was already somewhat on the decline, and it is possible that the potency of the suppressive factor may be less at this stage than at earlier stages. In particular, the attempts to inhibit a normal HvG response by injection of unresponsive cells may have failed because the capacity of these cells to suppress has decreased with the ageing of the mouse.

A third factor which hindered progress was the fact that the colony of mice used as recipients in these experiments appeared to enter a phase of abnormal behaviour, displayed in a number of ways. For example, their reactivity in the HvG and GvH reactions was noticeably depressed. In five

successive experiments, a dose of 5×10^6 allogeneic lymphoid cells failed to give an index of greater than 1.5 by weights, whereas in earlier experiments such a dose resulted in an index of at least three. By other criteria, however, the mice appeared to be hyperactive, requiring larger doses of viable cells than in earlier years to induce full tolerance to A-strain skin grafts in CBA neonates. In one experiment, tolerance towards C57B1 skin grafts could not be achieved even by the administration of 70×10^6 - spleen and bone marrow cells. Over the last year or two increasing difficulty in inducing unresponsiveness in the adult has been observed. Treatment with antigen extract and ALS, which used to give a median survival time of sixty days for A-strain skin grafts, can now only be reproduced with the help of Bordetella pertussis, which increases the effectiveness of the ALS. Unresponsive animals are subject, too, to rejection crises about 10 days after grafting, at a time when previously the ALS treatment alone would have been sufficient to ensure retention of all the grafts. Visual inspection suggested that this kind of crisis was non-specific in origin, the appearance of scar formation being quite different. This "non-specific" breakdown of allografts has been described by others.[2] In some experiments, this trouble resulted in there being no long-term survivors at all from a batch of thirty treated mice, and the supply of unresponsive animals was restricted still further.

Fortunately, these problems presented themselves only towards the end of the work program, and there is every reason to believe that the results cited here refer to tests carried out on mice with normal reactivity. The homing test remained consistent throughout, as this did not rely on a cell-mediated reaction.

The state of unresponsiveness adheres neither to the classical idea of

tolerance, nor to enhancement, and may well require for its existence a delicate balance between both cellular and humoral arms of the immune response. The precise mechanism has proved to be less readily solved than was expected, but the work presented here has narrowed the field of study, and suggests a number of further lines of investigation. The system is also being explored by other workers in this laboratory, and no doubt these different approaches will soon converge to produce a valuable, clinical tool for the induction of specific unresponsiveness towards allografts.

Appendix

Pannet Compton's Solution (PC)

155.6 g NaCl

14.9 g KCl

13.2 g $CaCl_2.6H_2O$

12.2 g $MgCl_2.6H_2O$

Made up to 1 litre in distilled water.

Unless otherwise stated, the solution is buffered to pH 7.6 by addition of 5 ml phosphate buffer (containing 0.312 g/l $NaH_2PO_4.2H_2O$ and 3.2 g/l $Na_2HPO_4.2H_2O$) to 95 ml PC, giving Ringer Phosphate Solution.

Avidity of Unresponsive Serum

The proportion of antibody bound to cells in the experiment recorded in Table VIII can be estimated by reference to the titration curve of unresponsive serum in Figure I (Chapter I). According to this graph, the reduction of homing activity from 4.3 to 3.8 corresponds to a decrease in antibody concentration to 0.8 of the original value. The ratio of free:bound antibody is 0.8:0.4 (ie 4). The association constant for the antigen-antibody interaction is given by

$$K = \frac{[Ab\text{-}Ag]}{[Ab]\ [Ag]} = \frac{1}{4\,[Ag]}$$

Although the antigen concentration has not been measured directly, one can find the order of magnitude by considering the concentration of extra antibody required for the homing index to reach the plateau value (when the cells are completely covered). The curve has been extrapolated in the direction of the plateau in Figure I in such a way that the decrease in the homing index of EXP I from 6.5 to 5.2 after absorption corresponds to a smaller reduction in antibody concentration than for EXP II (since it is presumed that in EXP I there was less antigen available).The gradient is therefore very steep, and the antibody concentration required to attain the plateau value, from a homing index of 4.3, is within one ten-fold dilution.

In these circumstances, the molar concentration of antigen, per mole of antibody present, must range from 1 to 10, giving a binding constant of 0.25 or less. Although this is only a very approximate figure, the low value shows that the avidity of the antibodies tested must also be low.

One would predict that with antibodies of higher avidity the same coverage would be achieved (and hence the same homing index) with a very much smaller quantity of total antibody, so that the proportion bound would be higher, and the reduction in homing index would be much greater after absorption. It would have been valuable to have confirmed this prediction using low concentrations of hyperimmune antibody, but unfortunately this experiment was not performed.

References: Introduction

1.   Burnet, F.M., Fenner, F. in "The Production of Antibodies"
           Melbourne: Macmillan (1949).

2.   Kaliss, N. Cancer Research 18, p.992 (1958).
           Immunological Enhancement of Tumour Homografts in Mice.
           A review.

3.   Beverley, P.C.L., Brent, L., Brooks, C., Medawar, P.B., Simpson, E.
           Transpl. Proc. 5, p.679 (1973).
           In Vitro Reactivity of Lymphoid Cells from Tolerant Mice.

4.   French, M.E., Batchelor, J.R. Transpl. Rev. 13, p.115 (1972).
           Enhancement of Renal Allografts in Rats and Man.

5.   Baldwin, R.W., Embleton, M.J., Price, M.R. Int. J. Cancer 12, p.84
           (1973).
           Inhibition of Lymphocyte Cytotoxicity for Human Colon
           Carcinoma by Treatment with Solubilized Tumour Membrane
           Fractions.

6.   Batchelor, J.R., Ellis, F., French, M.E., Bewick, M., Cameron, J.S.,
           Ogg, C.S.   Lancet 2, p.1007 (1970).
           Immunological Enhancement of Human Kidney Graft.

7.   Davies, D.A.L., Alkins, B.J. Nature 247, p.294 (1974).
           What Abrogates Heart Transplant Rejection in Immunological
           Enhancement.

8.   Billingham, R.E., Brent, L., Medawar, P.B. Phil. Trans. Roy. Soc. Ser. B.
           239, p.357 (1956).
           Quantitative Studies on Tissue Transplantation Immunity.
           III. Actively Acquired Tolerance.

9.   Billingham, R.E., Brent, L. Phil. Trans. Roy. Soc. Ser. B. 242,
           p.439 (1959).
           Quantitative Studies on Tissue Transplantation Immunity.
           IV. Induction of Tolerance in Newborn Mice, and Studies on
           the Phenomenon of Runt Disease.

10.   Brent, L., Gowland, G. Nature 192, p.1265 (1961).
           Cellular Dose and Age of Host in the Induction of Tolerance.

11.   Brent, L., Gowland, G. Nature 196, p.1298 (1962).
           Induction of Tolerance of Skin Homografts in Immunologically
           Competent Mice.

12.   Billingham, R.E., Medawar, P.B.   J. Exp. Biol. 28, 385 (1951).
       The Technique of Free Skin Grafting in Mammals.

13.   Lance, E.M., Medawar, P.B.   Proc. Roy. Soc. Ser. B. 173, p.447 (1969).
       Quantitative Studies on Tissue Transplantation Immunity.

14.   Woodruff, M.F.A., Anderson, N.A.   Nature 200, p.702 (1963).
       Effect of Lymphocyte Depletion by Thoracic Duct Fistula and
       Administration of Antilymphocytic Serum on the Survival of Skin
       Homografts in Rats.

15.   Gray, J.G., Monaco, A.P. Russell, P.S.   Surg. Forum 15, p.142 (1964).
       Heterologous Mouse Antilymphocyte Serum to prolong Skin
       Homografts.

16.   Brent, L., Kilshaw, P.J.   Nature 227, p.898 (1970).
       Prolongation of Skin Allograft Survival with Spleen Extracts and
       Antilymphocyte Serum.

17.   Levey, R.H., Medawar, P.B.   Ann. N.Y. Acad. Sci. 129, p.164 (1966).
       Some Experiments on the Action of Antilymphoid Sera.

18.   Brent, L., Hansen, J.A., Kilshaw, P.J.   Transpl. Proc. 3:1 p.684 (1971).
       Unresponsiveness to Skin Allografts Induced by Tissue Extracts and
       Antilymphocyte Serum.

19.   Pinto, M., Brent, L., Thomas, A.V.   Transpl. 17, p.477 (1974).
       Specific Unresponsiveness to Skin Allografts in Mice.
       III. Synergistic Effect of Tissue Extracts, Bordetella Pertussis,
       and Antilymphocyte Serum.

20.   Kilshaw, P.J., Brent, L., Thomas, A.V.   Transpl. 17, p.57 (1974).
       Specific Unresponsiveness to Skin Allografts in Mice.
       II. The Mechanism of Unresponsiveness Induced by Tissue Extracts
       and Antilymphocyte Serum.

21.   Brent, L., Pinto, M.   in Immunological Aspects of Transplantation
       Surgery. (Ed. Roy Calne) M.T.P.
       Induction of Specific Unresponsiveness by Donor Antigen and
       Non-Specific Immunosuppression.

References - Chapter I

1.   Lance, E.M., Taub, R.N.   Nature New Biol. 221, p. 841 (1969).
        Segregation of Lymphocyte Populations through Differential
        Migration.

2.   Zatz, M.M., Lance, E.M.   Cell. Imm. 1, p.3 (1970).
        Distribution of Chromium 51-labelled Lymphoid Cells in the Mouse.
        A Survey of Anatomical Compartments.

3.   Taub, R.N., Lance, E.M.   Immunology 15, p.633 (1968).
        Effects of Heterologous Anti-lymphocyte Serum on the Distribution
        of ^{51}Cr-labelled Lymphoid Cells in Mice.

4.   Lance, E.M.   Clin. Exp. Imm. 6, p.799 (1970).
        The Selective Action of Anti-lymphocyte Serum on Recirculating
        Lymphocytes.   A Review of the Evidence and Alternatives.

5.   Goldschneider, I., McGregor, D.D.   J. Exp. Med. 127, p.155 (1968).
        Migration of Lymphocytes and Thymocytes in the Rat.
        I. Route of Migration from Blood to Spleen and Lymph Nodes.

6.   Taub, R.N.   Fed. Proc. 29, p.142 (1970).
        Effect of Heterologous Anti-lymphocyte Serum on Lymphoid Cells
        Labelled with ^{125}IUdR.

7.   Gillette, R.W., Lance, E.M.   J. Ret. Soc. 10, p.273 (1971).
        Kinetic Studies of Macrophages.   I. Distribution Characteristics
        of Radiolabelled Peritoneal Cells.

8.   Zatz, M.M., Gringrich, R., Lance, E.M.   Immunology 23, p.665 (1972).
        The Effect of Histocompatibility Antigens on Lymphocyte Migration
        in the Mouse.

9.   Zatz, M.M., Lance, E.M.   J. Exp. Med. 134, p.224 (1971).
        The Distribution of Chromium 51-labelled Lymphoid Cells in
        Antigen-Stimulated Mice.

10. Woodruff, J.J., Gesner, B.M.   J. Exp. Med. 129, p.551 (1969).
        The Effect of Neuraminidase on the Fate of Transfused
        Lymphocytes.

11. Zatz, M.M., Goldstein, A.L., Blumenfeld, O.O., White, A.
        Nat. New Biol. 240, p.252 (1972).
        Regulation of Normal and Leukaemic Lymphocyte Transformation
        and Recirculation by Sodium Periodate Oxidation, and Sodium
        Borohydride Reduction.

12. Heslop, B.F., Hardy, B.E. Transplantation 13, p.364 (1972).
    In Vivo Damage to Circulating Lymphoid Cells by Alloimmune
    Serum in Rats.

13. Zatz, M.M., Goldstein, A.L., White, A. J. Immunology 111, p.1514
    (1973).
    Lymphocyte Population of AKR/J Mice. I. Effect of Ageing
    on Migration Patterns, Response to PHA and Expression of
    Theta-antigen.

14. Zatz, M.M., Mellors, R.C., Lance, E.M. Clin. Exp. Imm. 8, p.491.
    (1970).
    Changes in Lymphoid Populations of Ageing CBA and NZB Mice.

15. Pepys, M. Nature New Biol. 237, p.157 (1972).
    The Role of Complement in Induction of the Allergic Response.

16. Müller-Eberhart, H.J. Progress in Immunology, p.551. Editor B.Amos.
    Published by Academic Press 1971.
    Biochemistry of Complement.

17. Dr. Vojin Šljivić. Personal communication.

18. Ford, W.L., Gowan, J.L. Proc. Roy. Soc. Ser. B. 168, p.244 (1967).
    Role of Lymphocytes in Antibody Formation. II. Influence of
    Lymphocyte Migration on Initiation of Antibody Formation in an
    Isolated Perfused Spleen.

19. Ford, W.L., Sedgely, M. International Meeting on Inflammation.
    Paris (1975).
    The Migration of Lymphocytes across the Vascular Endothelium
    (Abstract).

20. Bainbridge, D.R., Brent, L., Gowland, G. Transplantation
    4, p.138 (1966).   51
    Distribution of Allogeneic   Chromium-labelled
    Lymph Node Cells in Mice.

References - Chapter II

1. Ford, W.L., Burr, W., Simonsen, M. Transplantation 10, p.258 (1970).
    A Lymph Node Weight Assay for Graft-versus-Host Activity of
    Rat Lymphoid Cells.

2. Twist, V.W., Barnes, R.D. Transplantation 15, p.182 (1973).
    A Popliteal Weight Gain Assay for the Graft-versus-Host Reaction
    in Mice.

3. Salaman, M.H., Wedderburn, N., Festenstein, H., Huber, B.
        Transplantation 16, p.29 (1973).
    Detection of a Graft-versus-Host Reaction between Mice
    Compatible at the H-2 Locus.

4. Salaman, M.H., Turk, J.L., Wedderburn, N. Transplantation 16,
        p.583 (1973).
    Foreign Antigenicity in Tissues of Mice Infected with
    Lymphomagenic Virus. I. Antigenicity of Spleen Cells.

5. Korčáková, L., Hašková, V. Transplantation 18, p.347 (1974).
    Regional Lymph Node Activation in Mice by a Soluble Product
    Released During Contact of Normal Allogeneic Cells.

6. Yoshida, Y., Osmond, D.E. Transplantation 12, p.121 (1971).
    Graft-versus-Host activity of Rat Bone Marrow Fractions and
    Lymphoid Tissues Quantitated by the Popliteal Lymph Node Assay.

7. Kerckheart, J.A.M., Benner, R., Willers, J.M.N. Immunology 25,
        p.103 (1973).
    Cells Involved in the Graft-versus-Host Reaction in Vitro.

8. Miller, J.F.A.P., Mitchell, G.F., Weiss, N.S. Nature 214, p.992
        (1970).
    Cellular Basis of Immunological Defects in Thymectomised Mice.

9. Cantor, H., Mandell, M.A., Asofsky, R. J. Immunology 104, p.409
        (1970).
    Studies of Thoracic Duct Lymphocytes of Mice. II. A
    Quantitative Comparison of the Capacity of Thoracic Duct
    Lymphocytes and other Lymphoid Cells to Induce a Graft-versus-Host
    Reaction.

10. Agyris, B.F. Transplantation 17, p.387 (1974).
    The Role of Macrophages, Thymus and Bone-Marrow Derived
    Cells in the Graft-versus-Host Reactions.

11. Monie, H.J., Everett, N.B. Anatomical Record 179, p.19 (1974).
The Popliteal Lymph Node Assay for Graft-versus-Host Interaction
in Mice. 1. Location and Proliferation of Donor and Host
Cells within the Node.

12. Davies, A.J.S., Doak, S.M.W. Nature 187, p.610 (1960).
Fate of Homologous Adult Spleen Cells injected into Newborn
Mice.

13. Howard, J.G., Michie, D., Simonsen, M. Brit. J. Exp. Path. 42,
p.478 (1961).
Splenomegaly as a Host Response in Graft-versus-Host Disease.

14. Bonney, W., Feldbush, T. Transplantation 15, p.215 (1973).
Host Contribution to the Popliteal Lymph Node G-v-H Reaction
in Rats.

15. Blomgren, H. Clin.Exp. Imm. 8, p.279 (1971).
Studies on Proliferation of Thymus Cells injected into Syngeneic
or Allogeneic Irradiated Mice.

16. Sprent, J., Miller, J.F.A.P. Cell. Imm. 3, p.385 (1972).
Interaction of Thymus Lymphocytes with Histoincompatible
Cells II. Recirculating Lymphocytes Derived from Antigen
Activated Thymus Cells.

17. Elkins, W.L. Transplantation 9, p.273 (1970).
Specific and Non-Specific Lymphoid Cell Proliferation in the
Pathogenesis of Graft-versus-Host Reactions.

18. Hall, J.G., Morris, B. Quart. J. of Exp. Physiol. 47, p.360 (1963).
Output of Cells from the Popliteal Lymph Nodes of Sheep.

19. McConnell, I., Lachmann, P.J., Hobart, M.J. Nature 250, p.113
(1974).
Restoration of Specific Immunological Virginity.

20. Taub, R.N., Gershon, R.K. J. Immunology 108, p.377 (1972).
The Effect of Localized Injection of Adjuvant Material on the
Draining Lymph Node III. Thymus Dependence.

21. Emeson, E.E., Thursh, D.R. J. Exp. Med. 137, p.1293 (1973).
Mechanism of Graft-versus-Host Induced Lymphadenopathy in
Mice. Trapping versus Proliferation.

22. Oppltová, L., Démant, P. Transplantation Proc. V.4 p.1367 (1973).
Genetic Determinants for the Graft-vs-Host Reaction in the
H-2 Complex.

23. Nabholtz, N., Vives, J., Young, H.M., Meo, T., Miggiano, V., Rijnbeck, A., Schreffler, D.C.   Eur. J. Imm. 4, p.378 (1974). Cell-mediated Cell Lysis in Vitro: Genetic Control of Killer Cell Production and Target Cell Specificities in the Mouse.

24. Schendel, D.J., Alter, B.J., Bach, F.H.   Transpl. Proc. V.4 p.1651 (1973). The Involvement of LD and SD-region Differences in MLC and CML: A Three Cell Experiment.

25. Pritchard, H., Micklem, H.S.   Clin. Exp. Imm. 10, p.151 (1972). Immune Responses in Congenically Thymus-less Mice. I. Absence of Response to Oxazolone.

26. Taub, R.N., Lance, E.M.   Immunology 15, p.633 (1968). Effects of Heterologous Anti-Lymphocyte Serum on the Distribution of Chromium 51-labelled Lymph Node Cells in Mice.

27. Snell, G.D., Hoeker, G., Amos, D.B., Stimpfling, J.H. Transplantation 2, p.777 (1964). A Revised Nomenclature for the H-2 Locus of the Mouse.

28. Gershon, R.K., Cohen, P., Hencin, R., Liebhaber, S.A. J. Immunology 108, p.586 (1972). Suppressor T Cells.

29. Zembala, M., Asherson, G.L.   Nature 244, p.227 (1973). Depression of the T-cell Phenomenon of Contact Sensitivity by T.cells from Unresponsive Mice.

30. Brooks, C.G., Brent, L., Kilshaw, P.J., New, R.R.C., Pinto, M., Transplantation 19, p.134 (1974). Specific Unresponsiveness to Skin Allografts in Mice. IV. Immunological Reactivity of Mice Treated with Liver Extracts, B. pertussis and Anti-lymphocyte Serum.

31. Kilshaw, P.J., Brent, L., Pinto, M.   Nature 255, p.489 (1975). Suppressor T-cells in Mice Made Unresponsive to Skin Allografts.

32. Elkins, W.L.   Transplantation 11, p.55 (1971). Sources of Immunogenic Stimulation of Lymphoid Cells Mediating a Local Graft-versus-Host Reaction in Rat Kidney.

33. Brent, L., Kilshaw, P.J. Nature 227, p.898 (1970). Prolongation of Skin Allograft Survival with Spleen Extracts and Anti-lymphocyte Serum.

References - Chapter III

1. Peterson, E.A., Sober, H.A. J. Am. Chem. Soc. $\underline{78}$, p.751 (1956).
    Chromatography of Proteins. I. Cellulose Ion-exchange
    Adsorbents.

2. Sober, H.A., Gutter, F.J., Wyckoff, M.M., Peterson, E.A.
    J. Am. Chem. Soc. $\underline{78}$, p.756 (1956).
    Chromatography of Proteins. II Fractionation of Serum Proteins
    on Anion Exchange Cellulose.

3. Heide, K., Schwick, H.G. in Handbook of Experimental Immunology
    Ch. 6. Ed. Weir, D.M. Published by Blackwell Scientific
    Publications.
    Salt Fractionation of Immunoglobulins.

4. Kalpaktsoglou, P.K., Hong, R., Good, R.A. Immunology $\underline{24}$,
    p.303 (1973).
    The Five Classes of Immunoglobulins in Normal C3H and
    Balb/c Mice.

5. Fahey, J.L., McLaughlin, C. J. Imm. $\underline{91}$, p.484 (1963).
    Preparation of Antisera Specific for 6-6Sγ Globulin
    β_{2c}-globulins, γ_1 macroglobulins and for Type I and II
    common γ-globulin determinants.

6. Fahey, J.L., Wunderlich, J., Mishell, R. J. Exp. Med. $\underline{120}$,
    p.223 (1964).
    The Immunoglobulins of Mice. I. Four Major Classes of
    Immunoglobulins 7Sγ 2-, 7Sγ 1-, γ 1A (β_2A)-, 18Sγ IM-globulins.

7. Dissanayake, S., Hay, F.C. Immunochem. $\underline{12}$, p.101 (1975).
    Isolation of Pure Normal IgG 1 from Mouse Serum.

8. Tiselius, A., Porath, J., Albertsson, P.A. Science $\underline{141}$, p.13 (1963).
    Separation and Fractionation of Macromolecules and Particles.

9. Onyewotu, I., Holborow, E.J., Johnson, G.D. Nature $\underline{248}$,
    p.156 (1974).
    Detection and Radio-assay of Soluble Circulating Immune
    Complexes Using Guinea-pig Peritoneal Exudate Cells.

10. Hunter, W.M., Greenwood, F.C. Nature $\underline{194}$, p.495 (1962).
    Preparation of Iodine - 131 labelled Human Growth Hormone of
    High Specific Activity.

11. McKearn, T.J., Hamada, Y., Stuart, F.P., Fitch, F.W. Nature
    $\underline{251}$, p.648 (1974).
    Anti-receptor Antibody and Resistance to Graft-versus-Host
    Disease.

12. Binz, H., Lindemann, J., Wigzell, H. Nature 246, p.146 (1973).
        Anti-Alloantibody Inhibition of a Local Graft-versus-Host
        Reaction.

13. Ramseier, H., Lindemann, J. J.E.M. 134, p.1083 (1971).
        Cellular Receptors. Effect of Anti-Alloantiserum on the
        Recognition of Transplantation Antigens.

14. Binz, H., Lindemann, J. J.E.M. 136, p.872 (1972).
        Cellular Receptors. Binding of Radioactively labelled
        Anti-Alloantiserum.

15. Lucas, Z.J., Enomoto, K., Zoltan, J. Fed. Proc. 32, p.4228 (1973).
        Enhancement of Renal Grafts by Anti-receptor Site Serum.

References: Summary and Conclusion

1.   Brooks, C.G., Brent, L., Kilshaw, P.J., New, R.R.C., Pinto, M.
          Transpl. 19, p.134 (1975).
          Specific Unresponsiveness to Skin Allografts in Mice:
          IV Immunological Reactivity of Mice Treated with Liver Extracts,
          B. pertussis, and Antilymphocyte Serum.

2.   Anderson, S.G., Cooper, S. Transpl. 10, p.342 (1970).
          Plaques of Cutaneous Necrosis in Skin Homografted Mice.

SPECIFIC UNRESPONSIVENESS TO SKIN ALLOGRAFTS IN MICE

IV. Immunological Reactivity of Mice Treated with Liver Extracts, *Bordetella Pertussis*, and Antilymphocyte Serum [1]

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SUMMARY

The strain-specific unresponsiveness to H-2-incompatible skin allografts induced by treatment of adult mice with single inoculations of donor strain liver extract and *Bordetella pertussis* vaccine, as well as three doses of antilymphocyte serum, has been investigated by several in vivo and in vitro methods, with a view to elucidating its mechanism. Lymphoid cells from mice with long surviving skin grafts were found to be reactive in graft-versus-host assays (as measured by splenomegaly or popliteal lymph node enlargement), and mixed lymphocyte culture tests gave positive results. Attempts to cause lethal runting of F_1 hybrid mice injected at birth with spleen cells from unresponsive mice gave variable results. However, the injection of F_1 hybrid cells into the footpads of unresponsive animals failed to elicit a significant host-versus-graft response. Although lymphoid cells from unresponsive animals did not include detectable numbers of cytotoxic cells, such cells could be generated by previous in vitro mixed lymphocyte culture stimulation or, to some degree, by the injection of the animals with F_1 hybrid cells. Attempts to prevent mixed lymphocyte culture stimulation or cytotoxicity with serum from unresponsive mice failed at the serum concentrations used. The data indicate that long-term unresponsiveness in this system is maintained by the production in the hosts of factors that interfere with the cell-mediated response.

Previous work (*4, 5, 26*) established the conditions in which a strictly limited treatment of adult CBA mice created a highly specific unresponsiveness to A/J skin allografts. Optimally, this entailed the use of donor strain tissue extracts, *Bordetella pertussis* vaccine, and antilymphocyte serum (ALS), as shown in Table 1. Our previously published data concerned with the mechanism of this unresponsiveness (*22*) were based on mice which had been made unresponsive without the aid of *B. pertussis*. We have no reasons for believing that the unresponsiveness of those animals differed from that of *B. pertussis*-treated mice in any sense other than a quantitative one, and inasmuch as neither classical tolerance nor en-

hancement could account for all the facts, we suggested the intervention of antigen-antibody complexes (*7, 22*). The experiments described here were intended to analyse more exhaustively the reactivity of mice with stable long-term unresponsiveness, with use of both in vitro and in vivo methods. Our results, taken together with the data reported elsewhere (*6, 21*), strongly support the notion that the cell-mediated responses of these mice are actively (and specifically) suppressed. While antigen-antibody complexes strongly contend for the role of mediator, we cannot at this stage rule out the possibility that suppressor cells, possibly of T cell origin, are involved.

MATERIALS AND METHODS

Mice, Skin Grafting, B. pertussis, Preparation of Liver Extracts, and ALS

For details of these procedures, see references *4* and *26*. CBA males were used through-

TABLE 1. Experimental design of induction of unresponsiveness in CBA males to donor strain skin allografts

Day [a]					
−16	−2	0	2	4	6
250 mg (wet weight) donor strain liver extract (i.v.)	0.2 ml *B. pertussis* (i.v.)	Skin graft	0.5 ml ALS (i.p.)	0.5 ml ALS (i.p.)	0.5 ml ALS (i.p.)

[a] Relative to the time of skin transplantation.

out as graft recipients, and unresponsiveness was induced for either A/J, (A), C57BL, or DBA/2 skin allografts. The basic experimental design for the production of unresponsive mice is given in Table 1.

Mouse Sera

All sera from unresponsive, control, or normal mice were prepared from blood taken from the heart. When not used fresh, sera were placed in sealed glass ampules and stored at −30 or −80 C, mostly at the latter temperature.

In Vitro Assays

Mixed lymphocyte culture (MLC). Lymph nodes or spleens were teased apart in fetal calf serum (FCS) on a fine meshed sieve and mashed gently. Cells were rinsed through into Universal containers (Sterilin) and washed 2 or 3 times in normal saline with 2% FCS. The cells were then suspended in RPMI-1640 (Grand Island Biological Company) without glutamine but containing 125 μg/ml ampicillin and cloxacillin, 2.0 mg/ml sodium bicarbonate, 15 mM HEPES, and FCS (all comprising the "medium"). For cultures in which no mouse serum had to be included the concentration of FCS was 10%, whereas for cultures with additional mouse serum (i.e., in inhibition experiments) 20% FCS was used to "buffer" the nonspecific effects of the mouse serum. Assessment of the number of viable cells was performed by phase contrast microscopy.

Where nonirradiated F_1 hybrid cells were used as stimulators, both responder and stimulator cell suspensions were adjusted to 5 × 10^6 viable cells/ml. Equal volumes of both suspensions were mixed, and 200-μl aliquots of mixed cells, stimulator cells, or responder cells were dispensed into the wells of Falcon Micro-

test II plates. All cultures were replicated 4 times. An additional control was established by culturing responder cells with syngeneic cells which were matched for age and sex with the F_1 stimulator cells; the small "responses" usually found in syngeneic cultures were subtracted from those of the mixed cultures, thus giving a net response to allogeneic antigens.

Where irradiated allogeneic stimulator cells were used, equal volumes of responder cells at 10 × 10^6/ml and irradiated cells at 5 × 10^6/ml were mixed, giving a 2:1 ratio. Syngeneic control "stimulator" cells were matched for age and sex with the allogeneic stimulator cells, and both were given 2,000 rads of irradiation in vitro (a dose that abolished their own MLC responsiveness).

Cultures were incubated in an atmosphere of 10% O_2, 4% CO_2, and 86% N_2 at 33–34 C; they were labelled after 3 or 4 days with methyl-^{3}H-thymidine (Amersham), each culture receiving 1.0–1.4 μc (specific activity 0.4 or 1.0 c/mmole). The cells were harvested 16–22 hr later and washed on paper discs by the method of Festenstein (*12*).

MLC responses are expressed as "stimulation" (i.e., the difference between the counts of mixed and control culture) and as "index of transformation" (i.e., the ratio of counts of mixed cultures to those of control cultures). Levels of significance were calculated by Student's *t*-test on log transformed data.

Cell-mediated lysis (CML) (16, 17). Lymph node cells were cultured in MLC with irradiated allogeneic or syngeneic cells for 4½ days; these cultures furnished populations of sensitised and control cells for the CML assay. The target cells were the C57BL EL4 lymphoma, the P-815-X2 DBA/2 mastocytoma, and an A strain lymphoma kindly provided by Dr. A. Boylston and Dr. K. T. Brunner. EL4

was passaged in vitro and the others in vivo. To label the target cells, approximately 2×10^6 cells were mixed with 200 μc of sodium chromate (^{51}Cr) (Amersham) in a total volume of 1.0 ml and incubated at 36–37 C for 80 min with mixing every 10 min. The labelled cells were washed twice with medium (see above), adjusted to between 5 and 10×10^4 cells/ml, and mixed with an equal volume of effector lymphocytes at a concentration giving the desired lymphocyte to target cell ratio. Aliquots of 200 μl of the mixture were dispensed into microtest plates and incubated at 30–37 C for approximately 6 hr (see Results). Each suspension provided three to four replicates. The cells were then resuspended gently with an Eppendorf pipette, transferred to 0.5- or 1.0-ml tubes, and spun down at 200 g for 10 min. The radioactivity in 100 μl of supernatant was measured. Maximum ^{51}Cr release was determined by freezing and thawing culture replicates 3–4 times, followed by centrifugation and counting of 100 μl of supernatant. The maximum release was 80–90% of total counts.

The percentage of cytotoxicity was calculated as follows (8):

$$\text{Cytotoxicity (\%)} = \frac{\begin{array}{c}{}^{51}Cr \text{ release in} \\ \text{the presence of} \\ \text{sensitised cells}\end{array} - \begin{array}{c}{}^{51}Cr \text{ release in} \\ \text{the presence of} \\ \text{control cells}\end{array}}{\begin{array}{c}{}^{51}Cr \text{ release} \\ \text{after freeze-} \\ \text{thawing}\end{array} - \begin{array}{c}{}^{51}Cr \text{ release in} \\ \text{the presence of} \\ \text{control cells}\end{array}} \times 100$$

Essentially the same method was used in measuring the cytotoxic responses of lymphocytes taken directly from unresponsive animals, with or without attempted in vivo stimulation by injecting F_1 hybrid spleen cells i.p.

Graft-Versus-Host (GVH) Assays

Splenomegaly. Spleen cells from unresponsive mice ("unresponsive" cells) were tested for antidonor strain reactivity by measuring the degree of splenomegaly 8 days after the inoculation of cells into F_1 hybrid newborns, as described elsewhere (22). Spleen indices were calculated in the usual way (28). Serum from unresponsive mice was used in attempts to inhibit GVH reactivity; it was stored at −80 C.

Popliteal lymph node weight gain assay (11). Peripheral and mesenteric lymph nodes from two to three unresponsive mice were

pooled, gently pressed through a mesh, and suspended in 20 ml of phosphate-buffered Ringer's solution containing 10% fetal calf serum. The cells were washed twice and resuspended in 10 ml of medium. Clumps were allowed to settle out by gravity over a period of 20 min. The supernatant contained single cells only, and after counting their concentration was adjusted to 2.5×10^8/ml. Twenty microliters of this suspension were injected into one of the hind footpads of adult F_1 hybrid mice; the contralateral footpads were injected with either F_1 or normal cells.

The cell recipients were killed after 6 days and the popliteal lymph nodes were removed (free of fat) and weighed accurate to 0.05 mg in a closed vessel. Results were expressed as the ratio of the weight of nodes draining feet injected with unresponsive or normal cells to the weight of nodes draining feet injected with F_1 hybrid cells.

Host-Versus-Graft (HVG) Assay

This assay is essentially the same as the popliteal lymph node weight gain assay already referred to, except that here F_1 hybrid lymph node cells were injected into the footpads of control or unresponsive mice. It has previously been shown that in this situation the draining nodes of normal mice enlarge and increase in weight as the result of HVG response (23, 27); it is therefore an appropriate test for measuring the reactivity of unresponsive mice.

Accordingly, the hind footpads of five unresponsive mice were injected with 10^7 (CBA $\times$ A)F_1 lymph node cells, while the contralateral footpads were inoculated with the same number of unresponsive CBA cells. In the control animals, which were age-matched mice treated with *B. pertussis* and ALS only, a comparison was made between F_1 cells and normal CBA cells. By this experimental design we ensured that the syngeneic cells injected into the contralateral footpads had the same immunological status as the hosts, and that a contribution by the syngeneic cells in the response against the F_1 cells can be ruled out.

RESULTS

In Vitro Reactivity of Lymphoid Cells

MLC. Lymph node cells taken from mice with long surviving grafts were generally

found to be reactive in MLC to donor strain histocompatibility antigens (Tables 2 and 3) as well as to third-party antigens (Table 2). The cells from two mice (Table 2, experiment 1) were significantly hyperreactive compared with normal mice (0.01 < *P* < 0.001); some had normal reactivity (Table 3, experiments 2 and 4) and the remainder responded somewhat more weakly. There can be little doubt, therefore, that unresponsive mice carrying entirely healthy skin allografts have lymphocytes that have the potential for recognising donor strain antigens and responding to them.

Cytotoxic assay. It is equally clear that unresponsive mice lack cells that are cytotoxic in short-term culture with specific target cells (first line of each of the experiments summarized in Table 4). However, when the same cells were tested in CML (i.e., after previous stimulation in MLC), the number of cytotoxic cells was virtually the same as in the stimulated control suspensions (Table 4, experiment 1). Cytotoxic cells can therefore be readily generated in vitro.

In order to answer the question of whether cytotoxic cells can be generated in vivo if antigenic stimulation is provided in addition to whatever antigens may emanate from the skin grafts, unresponsive mice were injected with a single dose of semiallogeneic spleen cells and assayed for the presence of cytotoxic cells 5

days later. Although cytotoxic cells were now present, their in vitro reactivity was only about one-half of that generated in the control mice (Table 4, experiments 2 and 3).

Reactivity of Lymphoid Cells in GVH Assays

Popliteal lymph node weight gain assay. The data presented in Table 5 show that lymph node cells from unresponsive mice were as responsive as cells from normal mice. In experiment 2 normal and unresponsive cells are compared with F_1 cells injected into opposite footpads of F_1 hybrid hosts; in experiment 1 an equally valid comparison is made between normal and F_1 cells on the one hand, and unresponsive and normal cells on the other. In neither case was the difference in reactivity between normal and unresponsive cells significant.

Splenomegaly. We have already shown (22) that spleen cells from unresponsive mice removed 40 days after transplantation react normally in the splenomegaly assay, but the mice used then had been made unresponsive by treatment with extract and ALS only (i.e., without the intervention of *B. pertussis*). Litters 3 and 4 in Table 6 entirely confirm the results with cells taken from donors that had carried healthy skin allografts for 3–6 months.

Runting. The question of whether unresponsive cells injected into newborn F_1 hybrid hosts can initiate the full sequence of events leading

TABLE 2. MLC responses to $(CBA \times A)F_1$ and $(CBA \times DBA/2)F_1$ stimulator cells by lymph node cells from CBA mice unresponsive to strain A skin allografts [a]

Experiment No.	Responder cells (CBA)		Mean background of unmixed cultures ± SE	Stimulation by $(CBA \times A)F_1$ cells ± SE		Index to $(CBA \times A)F_1$ ± SE	Index to $(CBA \times DBA/2)F_1$ ± SE
1	Normal		3,630 ± 120	1,610 ±	580	1.44 ± 0.15 [b]	13.9 ± 0.8
	Unresponsive	1	4,130 ± 230	5,480 ±	590	2.32 ± 0.17 [c]	12.1 ± 0.9
	Unresponsive	2	3,300 ± 190	3,560 ±	860	2.08 ± 0.25 [c]	15.9 ± 1.5
2	Normal	1	6,570 ± 530	4,260 ±	990	1.65 ± 0.18 [c]	9.4 ± 1.3
	Normal	2	4,660 ± 360	2,700 ±	740	1.60 ± 0.20 [c]	5.8 ± 1.2
	Unresponsive	1	15,100 ± 680	7,700 ± 1,500		1.51 ± 0.11 [c]	4.7 ± 0.4
	Unresponsive	2	6,330 ± 390	1,680 ±	840	1.23 ± 0.14 [d]	6.2 ± 0.7

[a] The figures refer to cell populations from individual mice; standard errors (SE) are given. Normal mice were matched for sex and age. Unresponsive mice had carried healthy skin grafts for 8 (experiment 1) or 5 (experiment 2) months.

[b] Level of significance of response in mixed culture: 0.01 > *P* > 0.001.

[c] Level of significance of response in mixed culture: 0.001 > *P*.

[d] Level of significance of response in mixed culture: 0.05 > *P* > 0.01.

TABLE 3. MLC responses to (CBA × C57BL)F₁ stimulator cells by lymph node or spleen cells pooled from CBA mice unresponsive to C57BL skin allografts [a]

Experiment No.	Responder cells (CBA)	No. of mice in pool	Mean background of unmixed cultures ± SE	Stimulation by (CBA × C57BL)F₁ ± SE	Index ± SE	Levels of significance [b]
1	Control (lymph node)	2	19,000 ± 750	57,900 ± 1,800	4.04 ± 0.18	
	Unresponsive (lymph node)	2	31,500 ± 900	57,500 ± 2,500	2.82 ± 0.11	$P < 0.001$
	Control (spleen)	2	16,900 ± 700	21,100 ± 1,400	2.25 ± 0.12	
	Unresponsive (spleen)	2	16,600 ± 300	15,700 ± 1,100	1.94 ± 0.07	$0.05 > P > 0.01$
2	Normal (lymph node)	1	12,400 ± 170	22,900 ± 1,500	2.81 ± 0.13	
	Unresponsive (lymph node)	1	14,610 ± 660	20,700 ± 2,600	2.42 ± 0.20	NS
3	Normal (lymph node)	3	5,340 ± 220	31,600 ± 700	6.92 ± 0.32	
	Unresponsive (lymph node)	3	6,860 ± 280	24,700 ± 1,000	4.61 ± 0.26	$P < 0.001$
4 [c]	Control (lymph node)	3	3,880 ± 140	5,580 ± 270	2.44 ± 0.11	
	Unresponsive (lymph node)	2	4,830 ± 260	6,060 ± 370	2.25 ± 0.13	NS

[a] The unresponsive mice had carried healthy skin grafts for 2 (experiments 1, 3, and 4) or 9 (experiment 2) months. Control mice had been injected with *B. pertussis* and ALS only.

[b] NS, not significant.

[c] In experiment 4 control and unresponsive lymph node cells were cultured with irradiated CBA and C57BL spleen cells.

TABLE 4. Generation of cytotoxic cells (CML) following in vitro or in vivo stimulation of lymph node cells from CBA mice unresponsive to C57BL skin allografts [a]

Experiment No.	Mode of stimulation	Effector cells	Stimulator spleen cells	Killing of EL4 (C57BL) cells (% ± SE)	Killing of P-815-X2 (DBA/2) cells (% ± SE)
1 [b]	None	Unresponsive	—	1.7 ± 0.3	1.3 ± 0.8
	In vitro	Control	(CBA × C57BL)F₁	57.0 ± 3.0	14.0 ± 2.0
		Unresponsive	(CBA × C57BL)F₁	56.0 ± 3.0	13.0 ± 2.0
		Unresponsive	(CBA × DBA/2)F₁	6.0 ± 1.0	83.0 ± 8.0
2 [c]	None	Unresponsive	—	−0.4 ± 0.6	ND [e]
	In vivo	Control	(CBA × C57BL)F₁	29.0 ± 1.0	ND
		Unresponsive	(CBA × C57BL)F₁	11.0 ± 1.0	ND
		Control	(CBA × DBA/2)F₁	ND	45.0 ± 2.0
		Unresponsive	(CBA × DBA/2)F₁	ND	46.0 ± 1.0
3 [d]	None	Unresponsive	—	0.8 ± 1.0	ND
	In vivo	Unresponsive 1	(CBA × C57BL)F₁	39.0 ± 1.0	ND
		Unresponsive 2	(CBA × C57BL)F₁	42.0 ± 2.0	ND
		Unresponsive 3	(CBA × C57BL)F₁	52.0 ± 2.0	ND
		Control	(CBA × C57BL)F₁	78.0 ± 1.0	ND

[a] Control cells were obtained from age-matched mice treated with *B. pertussis* and ALS only.

[b] Cell pools were from two mice. Unresponsive mice had carried healthy grafts for 2 months. The responder to stimulator cell ratio was 3:1 and MLC stimulation lasted 4½ days. The culture period for CML was 5½ hr and the effector to target cell ratio was 150:1. Effector cells were derived from lymph nodes.

[c] In vivo stimulation was attempted by injecting cell donors i.p. with 15 × 10⁶ F₁ spleen cells. Splenic effector cells were obtained 5 days later, washed once only, and cultured for 12 hr at an effector to target cell ratio of 200:1. Unresponsive mice had carried healthy grafts for 2 months.

[d] Conditions were the same as for experiment 2, except that the number of stimulator cells injected was 24 × 10⁶ and effector cells were washed 3 times.

[e] ND, not determined.

TABLE 5. Popliteal lymph node weight gain assay (GVH) of lymph node cells from normal CBA mice or from CBA mice made unresponsive to strain A skin allografts injected into the hind footpads of adult (CBA × A)F$_1$ hybrids[a]

Experi- ment No.	Site of cell injection		Ratio (with SE)
	Left side	Right side	
1A	Normal CBA	(CBA × A)F$_1$	Normal/F$_1$
	3.8	—	
	3.8	2.2	1.7
	2.5	2.2	1.1
	5.3	3.0	1.8
	3.2	1.8	1.8
Mean	3.7	2.3	1.6 ± 0.2
1B	Normal CBA	Unresponsive	Unresponsive/normal
	1.7	4.3	2.5
	1.6	2.6	1.6
	3.3	3.1	0.9
	2.4	1.4	0.6
	3.1	4.8	1.6
Mean	2.4	3.2	1.4 ± 0.3
2A	Normal CBA	(CBA × A)F$_1$	Normal/F$_1$
	3.0	1.4	2.1
	2.1	1.6	1.3
	2.7	1.5	1.8
Mean	2.6	1.5	1.8 ± 0.2
2B	Unresponsive	(CBA × A)F$_1$	Unresponsive/F$_1$
	3.1	1.2	2.5
	1.6	1.5	1.1
	2.5	1.7	1.5
	5.1	1.7	3.0
	4.6	1.8	2.5
Mean	3.4	1.6	2.1 ± 0.4

[a] The figures are the weights in milligrams of individual popliteal lymph nodes.

to runting and death was explored because it seemed possible that although able to undergo the early recognition phenomena involved in MLC transformation, further progression into full-fledged cytotoxic units was denied these cells. The results are somewhat tantalizing in that in two out of three experiments (Table 7) there was a distinct reduction in runting compared with normal cells. In the third experiment runting was, however, near normal.

Attempts to Inhibit In Vitro or In Vivo Reactivity of Normal and Unresponsive Cells with Serum from Unresponsive Mice

MLC. Neither normal (Table 8) nor unresponsive (Table 9) cells showed any measur-

able reduction in MLC responsiveness in the presence of serum from unresponsive mice tested at concentrations of up to 6%; hyperimmune serum, on the other hand, inhibited MLC responses of both kinds of responder cells very markedly (Tables 8 and 9). It was not possible to use higher concentrations of

TABLE 6. Attempt to inhibit GVH reactivity (splenomegaly assay) with serum from CBA mice unresponsive to strain A skin allografts[a]

Litter No.[b]	Normal cells and unresponsive serum		Normal cells and normal serum	
1	1.27		1.42	1.46
	1.50	1.38	1.51	
	1.36			
2	1.47	1.51	1.59	1.68
	1.56		1.78	

	Unresponsive cells and unresponsive serum		Unresponsive cells and normal serum	
3	1.55		1.66	
	1.72	1.60	1.37	1.48
	1.55		1.41	
4	1.57		1.52	1.52
	1.35	1.43	1.52	
	1.37			

[a] The figures are splenic indices for individual (CBA × A)F$_1$ littermates and their means 8 days after the injection of 10^7 CBA spleen cells.

[b] Litters 1 and 2: unresponsive serum from pool of unresponsive mice 3–6 months after skin transplantation. No haemagglutinating antibodies were detectable. Newborn (CBA × A)F$_1$ littermates were injected i.p. with 0.1 ml of serum and 3 hr later with 10^7 CBA spleen cells; on day 3 they received an additional dose of 0.1 ml of serum. Spleen indices in all experiments (including litters 3 and 4) were based on comparisons with littermates injected with normal serum and F$_1$ cells. Litters 3 and 4: unresponsive serum and cells from three mice 5 months after skin transplantation. Normal CBA cells were preincubated with unresponsive or normal serum (0.05 ml/10^7 cells) for 30 min and injected together with the serum. Additional doses of serum were given on days 1 (0.05 ml), 3 (0.04 ml), 4 (0.03 ml), and 6 (0.03 ml).

TABLE 7. Graft-versus-host reactivity (runting assay) of spleen cells taken from CBA mice unresponsive to C57BL skin allografts

Experiment No.	No. of (CBA × C57BL)F₁ litters	Cells injected (2 × 10⁷)	Proportion of recipients surviving on day 35[a]
1	1	F₁	2/2
		Unresponsive	3/3[b]
		Normal CBA	0/3
2	3	F₁	3/3
		Unresponsive	3/4[c]
		Normal CBA	1/6
3	2	F₁	5/5
		Unresponsive	1/4[d]
		Normal CBA	0/5

[a] No deaths occurred after day 35.

[b] Two out of three had subnormal body weight until about day 50.

[c] One out of four had subnormal body weight until about day 50.

[d] The sole survivor was grossly runted.

mouse serum because of its high toxicity to mouse cells in culture.

CML. For the shorter culture period of 6–7 hr used in this assay the problem of serum toxicity was not nearly as pressing, and mouse serum concentrations of up to 11% could be used without difficulty. Even so, no inhibition of cytotoxicity occurred with serum from unresponsive mice, whereas hyperimmune serum was highly effective (Table 10). Negative results were obtained regardless of whether unresponsive serum was present at either the MLC or the CML stage, or at both stages.

Splenomegaly. Although the doses of unresponsive serum injected into newborn mice were very high in relation to body weight, no inhibition of graft-versus-host reactivity was observed even when the cells were exposed to serum before inoculation (Table 6).

Our negative findings derive support from the fact that unresponsive sera at concentrations of 5% did not inhibit the formation of allorosettes (*15, 24*) between strain A erythrocytes and regional lymph node cells from CBA mice that had been sensitized by previous skin allografts.

HVG Reactivity in Unresponsive Mice as Measured by the Popliteal Lymph Node Weight Gain Assay

Although it can be argued that the presence of an intact skin allograft on a treated mouse is in itself indisputable evidence that the animal's immunological system cannot mount an effective attack against cells carrying the donor strain alloantigens, independent evidence for this was sought by the introduction of fresh antigen (in the form of a cell suspension) into unresponsive animals. The popliteal lymph node weight gain HVG assay, which depends upon the recognition of foreign (F₁ hybrid) antigens by hosts of one of the parental strains, seemed a highly appropriate test because it would at the same time suggest whether the in vivo block occurs at an early (antigen recognition) or late (generation of cytotoxic cells) stage of the response.

The results of this experiment show that unresponsive mice are not stimulated by freshly introduced cells, in contrast to control mice (Table 11).

One possible objection against the HVG data is that cellular antigen might have been removed from the popliteal lymph node through the intervention of serum factors (such as opsonins), thus preventing a local interaction with host lymphocytes. This possibility may be dismissed, however, because we have shown that labelled F₁ hybrid cells are present in the popliteal lymph nodes of unresponsive mice in the same numbers as labelled syngeneic cells.

DISCUSSION

We have shown that lymphoid cells taken from mice made unresponsive to H-2-incompatible skin allografts by treatment with donor strain liver extract, *B. pertussis,* and ALS are reactive to donor strain histocompatibility antigens when removed from their natural milieu. The in vitro tests showing this conclusively were MLC and CML, and all the in vivo GVH assays (splenomegaly, popliteal lymph node weight gain assay, runting) were positive. There is less certainty about the level of reactivity. MLC results on the whole suggest a very weak but fairly consistent hyporeactivity compared with normal cells, as do the runting

TABLE 8. Attempts to inhibit the MLC reactivity of normal CBA lymph node cells to (CBA × C57BL)F₁ stimulator cells with varying concentrations of serum from mice unresponsive to C57BL skin allografts[a]

Experiment No.	Serum	Concentration (%)	Mean background of unmixed culture ± SE	Stimulation by (CBA × C57BL)F₁ cells ± SE	Index ± SE
1[b]	Control	0.75	16,500 ± 440	51,300 ± 1,800	4.1 ± 0.2
	Unresponsive	0.75	15,300 ± 520	47,800 ± 830	4.1 ± 0.1
	Hyperimmune	0.75	14,200 ± 740	21,800 ± 660	2.7 ± 0.2
	Control	1.5	10,100 ± 490	45,700 ± 3,200	5.5 ± 0.4
	Unresponsive	1.5	11,400 ± 500	47,300 ± 2,300	5.2 ± 0.3
	Control	3.0	5,670 ± 390	32,800 ± 2,100	6.8 ± 0.6
	Unresponsive	3.0	4,970 ± 220	30,400 ± 1,800	7.1 ± 0.5
	Hyperimmune	3.0	3,770 ± 180	9,300 ± 940	3.8 ± 0.3
	Control	6.0	1,500 ± 100	16,800 ± 1,200	11.3 ± 1.1
	Unresponsive	6.0	1,630 ± 180	13,700 ± 2,000	9.4 ± 1.7
2[c]	Normal	0.3	154 ± 22	3,350 ± 500	22.8 ± 4.6
	Unresponsive	0.3	137 ± 12	2,720 ± 520	20.9 ± 4.8
	Normal	3.0	108 ± 14	1,460 ± 240	14.5 ± 2.8
	Unresponsive	3.0	95 ± 10	1,600 ± 170	17.9 ± 2.6
3[c]	Normal	0.3	5,410 ± 360	20,800 ± 1,600	4.8 ± 0.4
	Unresponsive	0.3	4,340 ± 240	23,000 ± 1,200	6.3 ± 0.4
	Normal	3.0	3,470 ± 300	16,300 ± 1,800	5.7 ± 0.6
	Unresponsive	3.0	2,930 ± 220	15,100 ± 1,700	6.2 ± 0.7

[a] In no case was the difference in stimulation or index between control and unresponsive sera significant whereas hyperimmune sera showed highly significant inhibition ($P < 0.001$).

[b] Sera were pooled from two mice in each experiment and stored overnight at 4 C. Unresponsive serum donors had carried healthy grafts for 2 months. Stimulator cells were splenic. Control serum was obtained from age-matched mice treated with *B. pertussis* and ALS only.

[c] Sera were from single mice and used fresh. Unresponsive mice had carried healthy grafts for 6½ (experiment 2) and 9 (experiment 3) months. Stimulator cells came from lymph nodes.

experiments; splenomegaly, popliteal lymph node GVH assay, and CML indicate near normal reactivity. Taking the results as a whole it is reasonable to conclude that the lymphocytes of unresponsive mice retain a near normal capacity for responding to donor strain antigens, but there could, nevertheless, be some special significance in the apparently subnormal reactivity shown in the runting assay (see below).

The HVG (popliteal lymph node weight gain) data, on the other hand, corroborate the observations on skin graft survival (i.e., that unresponsive lymphoid cells left in their natural milieu cannot be triggered into proliferation or bring about graft destruction). Clearly, an active restraint is being placed upon them,

and this restraint is not present after the cells have been removed, washed, cultured in vitro, or transferred to F₁ recipients. This conclusion is strongly supported by other data (*6, 7, 21*) showing that parabiosis of unresponsive and normal syngeneic mice confers a transient unresponsiveness on the normal partners, that it is not fully effective in breaking the unresponsiveness, and that unresponsiveness is transferrable to normal, sublethally irradiated, or ALS-treated syngeneic mice by unresponsive spleen cells.

A full discussion about the precise nature of this restraint will be deferred until this other evidence has been fully documented. Although it is by no means the only possible explana-

TABLE 9. Attempt to inhibit the MLC reactivity of pooled lymph node cells from three normal or unresponsive mice with serum from the unresponsive donors[a]

Responder cells	Serum	Mean background of unmixed cultures ± SE	Stimulation by (CBA × C57BL)F₁ cells ± SE	Index ±SE
Normal	Normal	580 ± 80	5,220 ± 300	10.0 ± 1.5
	Unresponsive	870 ± 40	7,530 ± 310	9.7 ± 0.7
	Hyperimmune	410 ± 50	820 ± 80	3.0 ± 0.4
Unresponsive	Normal	890 ± 60	6,710 ± 180	7.5 ± 0.5
	Unresponsive	1,360 ± 50	9,400 ± 400	7.9 ± 0.4
	Hyperimmune	910 ± 100	1,040 ± 160	2.1 ± 0.3

[a] Serum concentration was 6%. Fresh serum was obtained from three unresponsive CBA mice having perfect C57BL skin grafts for 7 weeks, and from two age- and sex-matched normal CBA mice. Lymph node cells from these mice were cultured in MLC with either normal, unresponsive, or hyperimmune serum. Stimulating cells were (CBA × C57BL)F₁ lymph node cells. The indices obtained with normal and unresponsive serum did not differ significantly; inhibition with hyperimmune serum was highly significant ($P < 0.001$).

tion, the concept of serum factors blocking or inhibiting the T cell response seemed the most plausible as well as the most readily explored. Accordingly, we examined the serum of unresponsive mice for such factors using three assays: MLC, CML, and GVH (splenomegaly). The results were consistently negative. It should, nevertheless, be borne in mind that the natural toxicity of mouse serum enforced strict limits on the serum concentrations used, especially in the MLC. Even so, concentrations of up to 6% might reasonably have been expected to have inhibited the MLC response (compare references *10, 11, 13,* and *25*).

While these observations seem to argue against a role for serum blocking factors, it is possible that very small concentrations of serum factors suffice to prevent rejection of well established skin grafts in long-term unresponsive mice. For this reason, experiments are now in progress to determine the blocking activity of sera taken from mice during the induction of unresponsiveness and from mice with long surviving grafts after fresh antigenic stimulation.

Free antibody seems to be an unlikely candidate for the suppressive factor in our unresponsive mice because hemagglutinating and cytotoxic antibodies are nearly always absent (*22*), even after additional antigenic stimulation (*6*), and only low concentrations of antidonor antibodies (mainly IgG₁) have been de-

tected (*21*). This argument is reinforced by our observation that hyperimmune sera were highly effective in inhibiting MLC and CML reactions (Tables 8–10), and also by the work of Jose et al. (*20*), who demonstrated that immune sera effectively inhibited in vivo GVH reactions.

Inhibition of responsiveness could be mediated by other factors, such as antigen-antibody complexes (*1, 29*) or suppressor T cells (*18, 19, 30*), and these alternative explanations will be considered in more detail elsewhere (*6*).

Whatever the precise nature of the restraint, it could occur either at the level of antigen recognition and cell activation (early) or at the level of generation of cytotoxic cells (late). That an early block must be operative is suggested by the negative HVG experiment and by the absence of cytotoxic cells in unresponsive mice that have not received additional stimulation. Further support for this stems from our observation (*6*) that the unresponsiveness of long-term unresponsive mice can be broken by 5×10^7 sensitised lymphoid cells, although graft rejection did occur rather slowly.

The existence of an early block does not by any means vitiate the possibility of an additional late block affecting any cells that may have sneaked through and developed into mature cytotoxic cells. The fact that in vivo stimulation with donor strain spleen cells generated

TABLE 10. Attempts to inhibit CML with serum from CBA mice unresponsive to C57BL or DBA/2 skin allografts during the period of stimulation (MLC), at the CML stage, or both[a]

Experiment No.	Effector cells	Serum in MLC	Serum in CML	Cytotoxicity (% ± SE)	
				EL4 cells	P-815-X2 mastocytoma cells
1[b]	Unresponsive[d]	None	None	0 ± 1.0	—
	Control	None	None	24 ± 1.0	—
	Unresponsive	None	None	13 ± 1.0	—
	Control	None	2.5% control	19 ± 1.0	—
	Control	None	2.5% unresponsive	18 ± 1.0	—
	Control	None	2.5% hyperimmune	5 ± 0.5	—
	Control	None	11.0% control	11 ± 1.0	—
	Control	None	11.0% unresponsive	13 ± 1.0	—
	Control	None	11.0% hyperimmune	1 ± 0.5	—
	Unresponsive	None	11.0% control	8 ± 2.0	—
	Unresponsive	None	11.0% unresponsive	8 ± 1.0	—
	Unresponsive	2.0% control	None	34 ± 0.5	—
	Unresponsive	2.0% unresponsive	None	42 ± 2.0	—
	Unresponsive	2.0% unresponsive	11.0% control	31 ± 2.0	—
	Unresponsive	2.0% unresponsive	11.0% unresponsive	35 ± 2.0	—
2[c]	Unresponsive[d]	None	None	—	0 ± 1
	Control	None	None	—	47 ± 1
	Unresponsive	None	None	—	46 ± 1
	Control	None	2.5% control	—	36 ± 1
	Control	None	2.5% unresponsive	—	41 ± 1
	Control	None	11.0% control	—	29 ± 1
	Control	None	11.0% unresponsive	—	24 ± 2
	Unresponsive	1.5% control	None	—	70 ± 1
	Unresponsive	1.5% unresponsive	None	—	64 ± 1
	Unresponsive	5.0% control	None	—	29 ± 1
	Unresponsive	5.0% unresponsive	None	—	35 ± 1

[a] Control cell and serum pools came from three age-matched CBA mice treated with *B. pertussis* and ALS only. The three mice that provided the unresponsive cell and serum pools had carried healthy grafts for 2½ (experiment 1) or 3 (experiment 2) months. MLC stimulation was brought about by culture with irradiated allogeneic spleen cells for 4½ days. MLC indices were 2.44 ± 0.11 and 2.25 ± 0.13 (experiment 1; see experiment 4 in Table 2) for control and unresponsive cells, respectively, and 2.34 ± 0.10 and 2.42 ± 0.06 for experiment 2. Serum was used fresh throughout, except that in experiment 1 the serum used at the CML stage had been stored at −20 C for 5 days. The ratio of effector to target cells in the cytotoxic assay was 50:1 and the incubation period was 7 (experiment 1) or 6 (experiment 2) hr.

[b] Mice were unresponsive to C57BL skin grafts.

[c] Mice were unresponsive to DBA/2 skin grafts.

[d] Irradiated syngeneic CBA spleen cells were used in this experiment in place of allogeneic cells.

cytotoxic cells, coupled with our finding that such a procedure does not prejudice the survival of the animals' skin grafts (6), does seem to point that way (as does the partial amelioration of the GVH reaction in the runting assay). While there can be no doubt that the extra stimulation did generate cytotoxic cells in these experiments, it should be noted that their activity was about one-half of that generated in the control animals (which had been treated with *B. pertussis* and ALS only). Because of the nature of the kinetics of in vitro cytotoxic reactions (9), it could well be that the 2-fold reduction of killing found with cells from unresponsive mice represents a much greater difference in the number of cytotoxic cells generated compared with those produced in control mice. This point requires additional investiga-

TABLE 11. Host-versus-graft reactivity (popliteal lymph node weight gain assay) in the popliteal lymph nodes of CBA mice unresponsive to strain A skin allografts 89 days after skin transplantation

Host	No. of mice	Cells injected [a]	Mean node weight (mg) $\pm$ SE	Mean ratio of F_1 to syngenic cells
Unresponsive	5	(CBA $\times$ A)F_1	2.4 $\pm$ 0.3	0.9 $\pm$ 0.1
		Unresponsive	2.7 $\pm$ 0.2	
Controls[b]	5	(CBA $\times$ A)F_1	3.9 $\pm$ 0.2	2.0 $\pm$ 0.2
		Normal	2.0 $\pm$ 0.1	

[a] Lymph node cells (10^7) were injected into opposite hind footpads.

[b] Injected with *B. pertussis* and ALS only.

tion. The survival of grafts in these mice could therefore be attributable to a suboptimal number of cytotoxic cells or, alternatively, it could be attributable to a restraint at this late stage.

Judged by all criteria used in this study, these unresponsive mice differ profoundly from neonatally injected tolerant mice (*2, 3*).

LITERATURE CITED

1. Baldwin RW, Price MR, Robins RA: 1972 Nature 238: 185
2. Beverley PCL, Brent L, Brooks C, et al: 1973 Transpl Proc 5: 697
3. Brent L, Brooks CG, Lubling N, et al: 1972 Transplantation 14: 382
4. Brent L, Hansen JA, Kilshaw PJ, et al: 1973 Transplantation 15: 160
5. Brent L, Kilshaw PJ: 1970 Nature 227: 901
6. Brent L, Kilshaw PJ, Pinto M: Transplantation (in press)
7. Brent L, Pinto M: 1973, p 317 *In* Calne R (ed). *Immunological aspects of transplantation surgery.* MTP Co, Lancaster, England
8. Brunner KT, Mauel J, Rudolf H, et al: 1968 Immunology 14: 181
9. Canty TG, Wunderlich JR: 1971 Transplantation 11: 111
10. Ceppellini R, Bonnard GG, Coppo F, et al: 1971 Transpl Proc 3: 63
11. Cerottini JC, Brunner KT: 1971, p 369 *In* Bloom BR, Glade PR (eds). *In vitro methods in cell-mediated immunity.* Academic Press, New York
12. Festenstein H: 1968 Lancet 1: 182
13. Fontaine J, Voisin GA: 1974 C R Acad Sci (Paris) 278D: 681
14. Ford WL, Burr W, Simonsen M: 1970 Transplantation 10: 258
15. Gluckman JC, Bansal SC, Wright PW, et al: 1974 Transplantation 17: 97
16. Häyry P, Anderson LC, Nordling S, et al: 1972 Transpl Rev 12: 91
17. Häyry P, Defendi V: 1970 Science 168: 133
18. Jacobson EB: 1973 Eur J Immunol 3: 619
19. Jacobson EB, Herzenberg LA, Riblet R, et al: 1972 J Exp Med 125: 1163
20. Jose DG, Stutmann O, Good RA: 1974 Transplantation 17: 211
21. Kilshaw PJ, Brent L, Pinto M: Transpl Proc (in press)
22. Kilshaw PJ, Brent L, Thomas AV: 1974 Transplantation 17: 57
23. Meyer II, Heron I: 1973 Acta Pathol Microbiol Scand B 81: 724
24. Micklem IIS, Asfi C, Staines NA, et al: 1970 Nature 227: 947
25. Milton JD, Mowbray JF, Ruszkiewicz M, et al: 1973 Transplantation 15: 579
26. Pinto M, Brent L, Thomas AV: 1974 Transplantation 17: 477
27. Salaman MII, Turk JL, Wedderburn T: 1973 Transplantation 16: 583
28. Simonsen M: 1962 Progr Allergy 6: 349
29. Sjögren IIO, Hellström I, Bansal SC, et al: 1971 Proc Nat Acad Sci 68: 1372
30. Zembala M, Asherson GL: 1973 Nature 244: 227

Studies on the Mechanism of Specific Unresponsiveness to Skin Allografts in Mice

P. J. Kilshaw, L. Brent, C. G. Brooks, R. R. C. New, and M. Pinto

PREVIOUS reports[1] have established that specific immunological unresponsiveness to H-2-incompatible skin allografts can be induced in adult CBA mice by treatment with a single injection of a crude extract of donor-strain liver combined with a dose of *Bordetella pertussis* vaccine and a short course of antilymphocytic serum (ALS). Optimally, liver extract was injected 16 days before skin transplantation (day −16), *B. pertussis* on day −2, and ALS on days +2, +4, and +6; 30%–50% of CBA male mice treated by this regimen retained healthy strain-A skin grafts for longer than 100 days. This paper summarizes the results of recent in vivo and in vitro experiments[2,3] designed to analyze the immunological status of mice bearing healthy allografts of long standing.

Hemagglutinating and cytotoxic antibodies specific for the donor strain were invariably absent from the serum of mice treated with liver antigen, *B. pertussis*, and ALS, regardless of the time of sampling. Nevertheless, antibodies were detectable using more sensitive techniques. For example, ^{51}Cr-labeled donor-strain lymphnode cells incubated with serum from unresponsive mice and injected intravenously into normal mice were cleared from the circulation by the liver. In addition, donor red cells or thymocytes pretreated with "unresponsive" serum bound small quantities of ^{125}I-labeled rabbit antibodies specific for mouse immunoglobulins. The alloantibodies detected by this technique belonged primarily to the IgG$_1$ subclass.

Attempts to increase the production of anti-donor antibodies in CBA mice unresponsive to strain-A skin grafts by repeated injections of (CBA × A) F$_1$ hybrid spleen cells have usually failed, nor was the survival of skin grafts impaired by this treatment. That mice bearing healthy allografts of long standing were unresponsive to graft antigens was further substantiated by the observation that the survival of fresh donor-strain grafts was prolonged or indefinite, and that an injection of hybrid lymph-node cells into the footpads failed to elicit a proliferative response in the popliteal lymph nodes. When skin grafts were removed from unresponsive mice reactivity returned within 1–2 weeks.

In contrast to the unresponsiveness shown by the whole animal, spleen and lymphnode cells removed from mice that had retained healthy skin grafts for long periods were reactive against donor antigens in the mixed-lymphocyte-culture test (MLC). The results of four MLC experiments are shown in Table 1. In all cases, cells taken from CBA mice made unresponsive to C57BL skin grafts were stimulated by donor antigens, although in experiments 1 and 3 the indices of stimulation were significantly lower than those given by cells from control mice. Immunological responsiveness to donor antigens was virtually normal when assessed by graft-vs.-host (GvH) assays.

Cells taken from the spleens and lymph nodes of unresponsive mice and cultured with ^{51}Cr-labeled donor-strain tumor target cells for 12 hr failed to show cytotoxicity, but when unresponsive cells were stimulated with donor antigens in MLC cytotoxic cells were generated. Cytotoxic cells specific for donor antigens could also be produced in

Table 1. MLC Responses to (CBA × C57BL) F₁ Stimulator Cells by Lymph-Node or Spleen Cells Pooled from CBA Mice Unresponsive to C57BL Skin Allografts

Exp.	Responder Cells (CBA)	No. of Mice in Pool	Mean Background of Unmixed Cultures ± SE	Stimulation by (CBA × C57BL) F₁ ± SE	Index ± SE
1	Control (lymph node)	2	19,000 ± 750	57,900 ± 1,800	4.048 0.18
	Unresponsive (lymph node)	2	31,500 ± 900	57,500 ± 2,500	2.82 ± 0.11
	Control (spleen)	2	16,900 ± 700	21,100 ± 1,400	2.25 ± 0.12
	Unresponsive (spleen)	2	16,600 ± 300	15,700 ± 1,100	1.94 ± 0.07
2	Normal (lymph node)	1	12,400 ± 170	22,900 ± 1,500	2.84 ± 0.13
	Unresponsive (lymph node)	1	14,610 ± 660	20,700 ± 2,600	2.42 ± 0.20
3	Normal (lymph node)	3	5,340 ± 220	31,600 ± 700	6.92 ± 0.32
	Unresponsive (lymph node)	3	6,850 ± 280	24,700 ± 1,000	4.61 ± 0.26
4	Control (lymph node)	3	3,880 ± 140	5,580 ± 270	2.44 ± 0.11
	Unresponsive (lymph node)	2	4,830 ± 260	6,060 ± 370	2.25 ± 0.13

The unresponsive mice had carried healthy skin grafts for 2 (exp. 1, 3, and 4) or 9 (exp. 2) months. Control mice had been injected with *B. pertussis* and ALS only.

In exp. 4, control and unresponsive lymph-node cells were cultured with irradiated CBA and C57BL spleen cells.

vivo by injecting unresponsive mice with 15 × 10⁶ F₁ hybrid spleen cells, but the level of cytotoxicity detected was only half that observed in control mice that had received similar antigenic stimulation.

Attempts to suppress reactivity against donor antigens with serum taken from unresponsive mice have so far failed. The passive transfer of as much as 1.5 ml of "unresponsive" serum to normal syngeneic recipients had no effect on the survival of donor-strain skin grafts. Negative results were also derived from attempts to inhibit GvH reactions in newborn mice with serum. In vitro, "unresponsive" serum has been incorporated in MLC at a concentration of up to 6% and in a cell-mediated lysis assay up to 11%, but this has had no specific inhibitory effect.

In contrast to these negative results, partial unresponsiveness has been adoptively transferred to normal, sublethally irradiated, or ALS-treated syngeneic recipients with spleen cells from unresponsive mice. Figure 1 shows the results of an experiment in which 10⁸ spleen cells from CBA mice made unresponsive to strain-A skin grafts were transferred to syngeneic recipients that had been irradiated with 400 R. The survival of strain-A grafts on these mice was sig-

nificantly prolonged compared with controls that had received cells from normal mice or irradiation only. In another experiment the ability of spleen cells to transfer unresponsiveness has been abolished by treatment with anti-θ antibodies and complement.
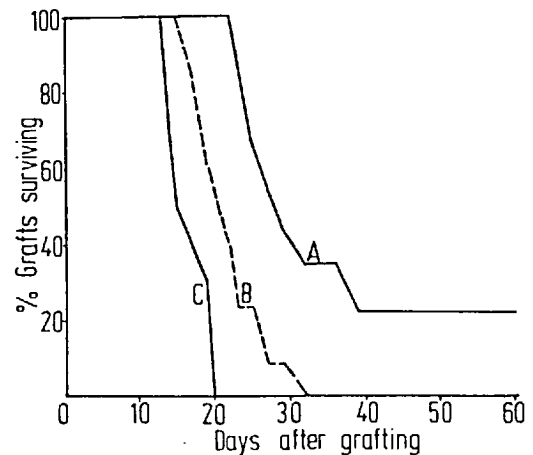
Fig. 1. Adoptive transfer of spleen cells from unresponsive mice to irradiated recipients. CBA moles were irradiated with 400 R and injected on the following day with 10⁸ spleen cells from unresponsive CBA mice bearing healthy strain-A skin grafts 110 days after transplantation. Control mice received normal spleen cells or irradiation only. One day later the mice were grafted with strain-A skin. A, unresponsive cells (9 mice); B, irradiation only (13 mice); C, normal cells (10 mice).

Immunological responsiveness in these mice, which had retained skin allografts for long periods, is clearly under active restraint. Corroborative evidence comes from the observation that treatment with cyclophosphamide 3 to 5 weeks after transplantation impaired rather than improved graft survival. The adoptive transfer of sensitized (but not normal) syngeneic cells to mice bearing well-established grafts led to rejection; this indicates that suppression is mainly afferent or central, inhibiting the generation of effector cells.

The data, so far, suggest that serum alloantibodies are not the inhibitory agent. Nevertheless, negative observations such as the failure of unresponsive serum to inhibit in vivo and in vitro reactions and the detection of only very low concentrations of antibodies in unresponsive mice are not entirely persuasive. We cannot completely rule out the possibility that antibodies in a critical concentration, perhaps complexed with antigens released from the graft, could play a suppressive role. A plausible alternative explanation at present under investigation is that suppressor T cells are responsible.

REFERENCES

1. Pinto M, Brent L: Transplantation 17:477 1974

2. Brooks CG, Brent L, Kilshaw PJ, et al: Transplantation (in press)

3. Brent L, Kilshaw PJ, Pinto M: Transplantation (in press).