# A STUDY OF THE DEVELOPMENT AND MAINTAINANCE OF IMMUNOLOGICAL RESPONSIVENESS IN MICE

by

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GENERAL INTRODUCTION.

The outstanding feature of lymphoid tissue is its extraordinary adaptive potentiality. It contains only a few morphological cell types, and these can secrete only a few basic types of protein molecule; yet, in response to stimulation with antigens, these antibody molecules can be impressed with any of a very large number (perhaps thousands) of discrete specificities. For this reason the immune response itself and immune tolerance have received a great deal of attention. Less attention has been paid to the ontogeny of lymphoid tissue, although this has many interesting features, and deserves study also for the light that may be thrown on some more general problems of immunity.

During the maturation of immune responsiveness (and probably throughout life) three important things must happen to developing lymphoid cells: they must aquire the competence to respond to foreign antigens; they must be prevented from responding to auto-antigens; and after first stimulation with antigen they must retain the "immunological memory" which enables them to give an enhanced response after subsequent stimulation. All these processes are taking place among cells which appear to be very much alike, and to have a fairly high overall rate of division: properties which on the face of it are hard to reconcile with the enormous range of possible specificities on the one hand, and with the long duration of tolerance and of immunological memory on the other. In the long term then, our need to understand the nature of immunological competence, tolerence and memory is likely to benefit from studies which

trace the development of these properties in the cells which manifest them. The more immediate problem is to characterise stages in the development of these cells, and to trace their lineage back to its embryonic origin.

A great impetus has been given to the study of the development of lymphoid tissue by the realisation of the vital part played in this process by the thymus. Many pointers are to be found in the older literature which would indicate that the thymus might play a central role in the lymphoid system, and that it would be a fruitful field of study. An extensive review of these will not be given here since there are good reviews by Miller, Marshall and White (1962); Miller (1963 a, b, 1964) and Arnason, Janković and Waksman (1962).

While the thymus has obvious lymphoid affinities, and is therefore likely to be concerned with immunity, it has certain remarkable features which distinguish it from a lymph node. Some of these are listed in Table I. From the information given in this table the following reasoning might be made about a possible immunological function of the thymus. Firstly, the poor performance of the thymus in immune reactions makes it unlikely that it contributes directly any important fraction to the overall immune responses of the body. And even if its cells were competent, they would be poorly placed to receive antigens since the thymus has neither the afferent lymph supply of lymph nodes nor the macrophage-lined venous sinusoids of the spleen. Neither is it likely that the thymus plays any important accessory role in immune reactions since these can take place almost equally well after, as before, thymectomy. If the thymus is in no way concerned with immune responses themselves, it could

Table 1. Summary of the salient points of difference between thymus and lymph node (taken from work published before 1961).

| Thymus | Lymph node | Reference |
|---|---|---|
| **Structure** | | |
| Division into cortex and medulla. | Follicular structure. | Maximow and Bloom, 1942. |
| Hassall's corpuscles. | None. | Maximow and Bloom, 1942. |
| None. | Germinal centres. | Maximow and Bloom, 1942. |
| "Blood-thymus barrier": macrophages did not take up vital stains (a) or proteins (b). | No barrier: macrophages take up these substances, injected i.v. | (a) Maximow and Bloom, 1942; (b) Coons, Leduc and Kaplan, 1951. |
| Thymus cells injected i.v. homed to spleen (a). | Thoracic duct lymphocytes homed to lymph nodes, not thymus (b) | (a) Fichtelius, 1953; (b) Gowans, 1959. |
| None has been found. | Afferent lymphatic drainage. | Greep, 1954. |
| Very high mitotic index (about 1 %). | Much lower (0.2 %) except during intense stimulation. | Andreasen and Christensen, 1949. |
| **Development** | | |
| Lymphocytes appear in early embryonic life. | Does not acquire lymphocytes until after thmus has done so. | Ball and Auerbach, 1960. |
| Extensive involution, atarting about the time of puberty. | Slight involution, starting later. | Andreasen, 1943. |
| **Immunological responses** | | |
| Very few plasma cells. | Many plasma cells. | Fagraeus, 1948. |
| No morphological response to systemic antigen injection. | Many changes, including increase in plasma cells. | Fagraeus and Gormsen, 1953. |
| Little or none. | Antibody formed in adoptive secondary response. | Harris, Harris and Farber, 1957. |
| Low activity in GVH reactions. | Highly active. | Vos, DeVries, Collenteur and Van Bekkum, 1959. |
| **Effect of extirpation** | | |
| Little or no effect in the adult. | No data available. | Harris, Rhodes and Stokes, 1948. |
| | Splenectomy inhibits some immune responses. | Rowley, 1950. |

still be concerned with the development of the lymphoid cells which are capable of performing these responses. The evidence from embryology had already suggested this; while the pattern of rapid growth and early involution clearly indicated that its function should be sought in early life.

A hint at the way in which this developmental function might be exerted could be found in the evidence from mitotic counts. The intense cell production by the thymus in early life, far outrunning the requirements for its own growth (Kindred, 1940), suggests that the thymus might be exporting the extra cells to other parts of the body - a possibility which found some support in the homing of transfused thymus cells to the spleen. Evidence that a different kind of controlling function might be in operation was found in the action of thymus extracts in increasing lymph node weight (Grégoire and Duchâteau, 1956; Comsa, 1957) or in stimulating peripheral lymphocytosis (Metcalf, 1958).

The possibility that the thymus might act by disseminating cells to the other lymphoid tissues prompted further speculations as to why the source of cells should be remote from their sites of action: perhaps it was necessary that during development they be insulated from contact with foreign antigens. Indeed, the absence of afferent lymphatics and the inability of thymus macrophages to take up proteins when these were administered intravenously, suggested that thymus cells are largely so insulated. If the development of tolerance to auto-antigens is an active process, akin to artificially-induced tolerance, then it might be an advantage for the developing cells to be

insulated from contact with foregin antigens so that they would not become tolerant of these also. At the same time, the cells must not become competent before the induction of auto-tolerance is complete. Here again it is possible (though not necessary) that this end might be gained if the developing cells were kept in a special environment where immunisation was somehow suppressed - perhaps by a local hormone.

These further speculations depend, however, on the correctness of the initial hypothesis that the thymus functions as a site for the maturation of lymphoid cells. There were two expectations from this hypothesis, which were thought to be most amenable to test:

1. Thymectomy, done sufficiently early in life should impair the development of immunological responsiveness.

2. If thymocytes are not competent while they remain within the thymus, or shortly after artificial release therefrom, they should become fully competent if given the right conditions.

Part II of this thesis describes some attempts to test these two expectations. It also describes experiments designed to trace the movements of cells into and out of the thymus. For these latter experiments it was necessary to have some means of marking cells, and for this the T6 chromosome marker was chosen. However, since the primary interest lay in lymphoid, and particularly in competent cells, it was an advantage to have a method of selectively stimulating division among these cells in order to reveal the chromosomes and the T6 marker. Part I describes such a method.

~~Since~~ The thymus continues active cell division throughout life, and thus it is reasonable to suppose that its function does not cease with immunological maturity, rather that it might continue to export cells and that these might serve to replace lymphoid cells lost in various ways. In Part III it is shown that the thymus is necessary in adult life for the recovery of immune function after this has been depressed by irradiation or paralysis, and that it is necessary for the maintainance of immunological responsiveness to its normal level.

## ASSUMPTIONS

There are certain basic problems for the solution of which there is at present little concrete evidence. Since the results to be described do not impinge directly on these problems, it was deemed simpler in the discussions to make certain tacit assumptions. The readjustments in the conclusions which would have to be made in order to suit the alternatives to these assumptions will in most cases be obvious.

(a) It has been assumed that a cell which has never been stimulated with antigen is multipotent with regard to the antibodies it can make. The alternative, or "clonal" hypothesis (Burnet, 1959) in which each cell is predetermined, would however suit most of the results equally well.

(b) It has been assumed that, once stimulated with an antigen, the range of further response of a cell, or its descendants, is restricted to that antigen.

(c) It has been assumed that the immunological potency of each cell in any particular immune reaction is the same. The alternative, that there exist "strong" cells and "weak" cells would greatly complicate the discussion and seems unwarranted at present.

(d) It has been assumed that immunological paralysis and tolerance to either tissue or other antigens are all facets of the same process. The word "paralysis" has been used throughout.

DEFINITIONS.

The following concepts have been used extensively, and require definition:

1. Stem cell - The least differentiated form in any series of cells capable of turnover or regeneration. They would be expected to have the greatest power to proliferate, and would thus be the chief source of new cells during regeneration.

2. Lymphoid stem cell - The stem cell at the base of the lymphoid series.

3. Competent cell - A cell previously unstimulated but in a state of readiness to respond to antigen, or antigen-adjuvant mixture (when these are presented in the correct form) by the production of antibody specifically reactive with the antigen.

4. Precursor cell - One which is not competent, but can become so without thymic influence. It differs in this respect from a lymphoid stem cell which, as will be discussed later, cannot differentiate further in the absence of the thymus.

5. Committed cell - Cell resulting from antigenic stimulation of a competent cell. It may be either (a) "active" in the production of antibody, or (b) an inactive "memory" cell.

6. Paralysed cell - One which is unable to respond (or to develop into a cell which can respond) to a specific antigen. It remains capable of response (or of development

into a cell capable of response) to unrelated antigens.

There is general acceptance of types 1 (Yoffey, 1960) and 3 (Medawar, 1963). That a cell can produce only one (or rarely two) types of antibody simultaneously, was shown by Attardi, Cohn, Horibata and Lennox (1959). Taking this with the long persistence of secondary responsiveness it is very probable that, once stimulated, a cell becomes irreversibly determined (Type 5). The existence of type 6 has not been proved, but would be required by assumption (a) above. Some attempt to characterise types 2 and 4 will be found in the ensuing pages.

7. Relative immunological potency (Potency), and

8. Relative immunological responsiveness (Responsiveness) - These refer to estimates of the relative numbers of competent cells in a cell suspension, and a whole animal respectively.

ABBREVIATIONS

| | | |
|---|---|---|
| ABC | - | Antigen binding capacity |
| BGG | - | Bovine gamma globulin |
| BSA | - | Bovine serum albumin |
| GVH | - | Graft-versus-host |
| NRS | - | Normal rabbit serum |
| PC | - | Proliferative capacity relative to normal adult bone marrow cells. |
| SRBC | - | Sheep red blood cells |
| STH | - | Salmonella typhi H. antigen |
| Tx | - | Thymectomised |
| Sh | - | Sham-operated |

## PART I: A METHOD FOR ESTIMATION OF THE PROPORTIONS CONTRIBUTED BY KARYOTYPICALLY-MARKED SYNGENEIC CELL POPULATIONS TO THE OVERALL IMMUNE RESPONSIVENESS OF MICE.

### INTRODUCTION.

The introduction of a visible chromosome translocation (T6) into an inbred strain of mice (CBA) has provided a very powerful tool for the study of cell migration (Carter, Lyon and Phillips, 1955). Unfortunately, chromosome markers can only be seen in dividing cells, and these are not necessarily representative of the whole population. Further, it is not possible in chromosome preparations to distinguish the types of cells which are dividing. In the present study the interest centres on lymphoid cells, and particularly on immunologically competent cells. The object of the method described below was to stimulate cell division among competent cells so that the dividing cells observed should be more representative of the lymphoid moiety within the tissues under examination. Stimulation of cell division was effected by injecting the pure strain cells into recipients which were an $F_1$ hybrid between the donor strain and another strain differing at the H-2 histocompatibility locus. In this situation, as is well known, a graft-versus-host (GVH) reaction ensues, along with much cell division, while the reaction of the host against the injected cells is genetically precluded. In order specifically to depress division of host cells, the recipients were irradiated immediately before the cell transfer (Howard, Michie and Simonsen, 1961).

Ideally, the method should measure the overall immune responsiveness at the same time as the contributions to this by various marked cell populations. The method would then be analogous to Simonsen's discriminant spleen assay (Simonsen, 1962) but it would have the difference that it would discriminate between syngeneic rather than allogeneic cell populations. One way to determine the overall responsiveness is to submit the lymphoid tissues to a GVH spleen weight assay as described later in this section - and this has been done in some experiements. Another way which is discussed later, would be to relate overall responsiveness to the mitotic response in the irradiated recipient.

Before starting work on chimaeras of CBA/T6T6 with CBA, however, it was necessary to be sure of adequate histocompatibility between these strains. This was tested in experiment 1, below.

The strong stimulation of mitosis during GVH reaction makes it likely that the majority of mitoses belong to cells specifically stimulated by most antigens. Nevertheless, mitoses will arise from other sources, and it is important to determine whether the contributions by these sources form important fractions of the total. The mitoses found in the host fall into four classes:

1. Those cells called into mitosis by the specific action of the host histocompatibility antigens (competent cells);

2. Those cells, playing some hypothetical accessory role, which might divide in a non-specific response to the already

existing GVH reaction;

3. Those cells whose rate of division is independent of the GVH reaction; and

4. Residual mitoses among the irradiated host cells.

It is easy to distinguish the host mitoses, and as will be seen from Experiment 2, they contribute only a very small proportion to the total. It is less easy to distinguish the other three classes. One way might be to inject marked hybrid cells - e.g. (CBA/T6T6 x C57BL)$F_1$, - which would be genetically incapable of GVH reaction - in order to represent classes 2.and 3. Unfortunately these cells would be treated by the CBA/T6T6 donor cells as part of the host, and thus would become a target of GVH reaction. In such a situation these cells, not being irradiated, might divide rapidly as they do in conventional GVH reaction (Davies and Doak, 1960). Experiment 2, described below, showed this to be the case. Another method of representing classes 2. and 3., by using cells from CBA/T6 mice previously made tolerant of C57BL antigens, would be equally impracticable since no way is at present known of inducing complete tolerance to histocompatibility antigens without also inducing a state of cellular chimaerism. While an intrinsic control for the donor mitoses not specifically dependent on the GVH reaction does not appear to be feasible, it is possible to make some estimate of the contribution by class 3 (donor mitoses independent of the GVH reaction) by using a separate host mouse, which is syngeneic with the donor cells. This was done in Experiments 3, and 4. These two experiments were also intended to determine the optimum cell dose and time after cell transfer, at which to make the chromosome preparations.

## MATERIALS AND METHODS.

Animals. Mice of the inbred strain CBA, and CBA/T6T6 and $F_1$ hybrids of CBA with C57BL/6 were used. The T6 translocation was first produced at the MRC Radiobiological Unit, Harwell, Berks. by means of irradiation. By repeated backcrossing this translocation was then introduced into the CBA inbred strain. These mice, homozygous for the translocation, are here designated CBA/T6T6, while heterozygotes produced by crossing CBA/T6T6 with CBA are designated CBA/T6. The karyotypes are designated T6T6 when both chromosomes are present, T6 when only one is present and T6- when neither is present. CBA/T6T6 mice came originally from Harwell, while CBA and C57BL mice came from University College, London.

Irradiation. The mice were irradiated in a circular box 20 cm. in diameter and 4 cm. deep. The conditions of irradiation were 125 kvp, 5.5 mA, and the filtration was 1 mm. aluminium plus the plastic lid of the box. The X-ray source was below the box which contained up to 10 mice in individual compartments separated by radial divisions. The distance between the target and a point 2 cm. above the floor of the box was 32 cm. The mean dose rate over the area occupied by the mice at 2 cm. above the floor of the box was about 40 r/min., but the machine was calibrated at the start of each day of use. The box was mounted on a revolving turntable so as to equalize the doses received by individual mice. Under these conditions the 50% lethal dose in 30 days was about 950 r.

spleen, lymph node (cervical, axillary, inguinal and mesente

Cell transfers. Suspensions of pooled ~~lymphoid~~ or bone marrow cells were usually prepared and injected as described by Billingham and Brent (1959) except that Gey's solution was used instead of Ringer-phosphate, and the cells were not washed unless they were to be injected intravenously. Where suspensions from individual mice were required, the chopped material of each mouse was put into a separate ground glass homogeniser of very loose fit (difference of at least 1 mm. in diameter between plunger and tube), containing 1-5 ml. of Gey's solution (Gey & Gey, 1936). The cells were dissociated by alternately pressing the fragments gently against the side, and then washing away the cells by moving the plunger up and down a few times. The suspension was put through a fine seive before use.

The proportion of viable cells in the suspensions was estimated by Shrek's eosin technique (Shrek 1936). Between 60% and 90% of the cells failed to take up the stain and were counted as "viable". Lymphoid cells were usually injected intraperitoneally, except where otherwise specified. Bone marrow cells were always injected intravenously.

Skin Grafts. These were applied and scored as described by Billingham and Medawar (1951), with slight modifications used by Dr. L. Brent (personal communication, 1962). Latterly, grafts of tail skin were used rather than body skin, since this simplified preparation of the grafts.

Graft-versus-host spleen weight assay. Immunological potentiality is usually estimated from the responses of the

animal itself to antigenic stimulus - as measured in terms of antibody production or allograft rejection for example. There is evidence, however, that these responses are normally limited by some kind of homoeostatic mechanism in such a way that increase in the quantity of lymphoid tissue above a certain level will not cause further increase in the response. For example, Makinodan, Kastenbaum and Peterson (1962) found that as little as 3.5% of the normal complement of spleen cells would produce a response of normal magnitude after transfer to recipients whose own immune responsiveness had been destroyed by irradiation. A quantitative estimate of responsiveness, therefore, can best be obtained in such a transfer system where sub-maximal responses can be arranged. One of these is the GVH spleen weight assay.

Parental strain lymphoid cells injected into $F_1$ hybrid hosts are known to react immunologically against the host, and in doing so to cause enlargement of the spleen. This reaction can be used as a measure of the immunological potency of a cell suspension (see Simonsen, 1962). For a critical comparison of the potency of two such cell suspensions an assay procedure must be used, since the spleen enlargement response cannot be taken as proportional to the cell dose except over a very restricted range. The 4-point spleen weight assay described below is slightly modified from that developed by Dr. D.Michie (personal communication, 1962).

One (C57BL x CBA)$F_1$ hybrid litter containing at least 5 mice aged 3-10 days was used for each assay. A mixed spleen

and lymph node cell suspension was prepared from each donor mouse and diluted to 100 million cells/ml. The "Test" suspension was made from the mouse whose immunological responsiveness was to be assessed, and the "Standard" suspension from a normal untreated mouse 3-10 months old. A third suspension ("Blank") was made from a (C57BL x CBA)$F_1$ hybrid mouse, and was used to dilute the other two. The 5 hybrid baby mice were injected intraperitoneally with a total of 10 or 20 million cells/gm. body weight according to the following scheme:-

| Recipient Mouse. | Test Cells. | Standard Cells. | Blank Cells. |
|---|---|---|---|
| $T_1$ | 100% | - | - |
| $T_2$ | 20% | - | 80% |
| $S_1$ | - | 100% | - |
| $S_2$ | - | 20% | 80% |
| B | - | - | 100% |

Sometimes, where a very low potency was expected in the test suspension, the proportions of standard cells injected to mice $S_1$ and $S_2$ were reduced to 20% and 4% (or even 10% and 2%) respectively, in order that the spleen enlargement responses should not be too widely separted as between test and standard. The mice were killed 8 days after injection and the spleen and body weights recorded.

The spleen enlargement of each of the mice receiving test or standard suspensions was calculated in relation to the relative spleen weight of the mouse which received only blank

cells, as follows:-

$$\text{Spleen Index} = \frac{\text{Spleen wt.}}{\text{Body wt.}}\,(\text{T or S}) \div \frac{\text{Spleen wt.}}{\text{Body wt.}}\,(\text{B})$$

The relative potency of test/standard cell suspensions was then calculated from the formula:

$$\text{Relative potency} = \text{Antilog}\left[\bar{x}_s - \bar{x}_t - \frac{\log S_1 + \log S_2 - \log T_1 - \log T_2}{2b}\right]$$

where $\bar{x}_s$, $\bar{x}_t$ are the mean log doses of standard and test cells injected, $S_1$, $S_2$, $T_1$, and $T_2$ are the responses in terms of spleen index, and b is a mean value of the slope of log response on log dose. Two estimates of b were obtained from each assay by the formulae, $b = \frac{\log T_1 - \log T_2}{0.699}$, or $b = \frac{\log S_1 - \log S_2}{0.699}$, and one mean value of b obtained from the average of these for each experimental group of assays.

To obtain an estimate of the number of competent cells in the test mouse as compared to the standard mouse ("relative responsiveness") the figure for the relative potency of the cell suspensions was multiplied by the fraction: total number of lymphoid cells (including non viable) obtained from test mouse/ total number of lymphoid cells obtained from the standard mouse.

Spleen indices were taken as significant only if they exceeded 1.32. This figure has been calculated to be significant at the 2.5% level for a comparison of one treated mouse with one control (receiving blank cells) by Michie,

Woodruff and Zeiss (1961). The doses of standard cells were usually high enough to ensure that the spleen indices exceeded this figure, but in some of the assays it was not possible to obtain enough cells from the thymectomised donors to ensure that both "Test" indices did so. These assays will overestimate the responsiveness of the "Test" mice.

Karyotype analysis.

1. Preparation.

Originally the method of Fox and Zeiss (1961). was tried, with various modifications, but it was finally abandoned in favour of a modification currently used by Ford (personal communication, 1963) of the drying method of Rothfels and Siminovitch (1958). This procedure was as follows:

The mouse was injected with 0.01 ml/gm body weight of an 0.4 mg/ml solution of Colcemid (Ciba) in saline intraperitoneally, and then killed 1 - 2 hr later. The organs to be examined were removed and teased individually in 5 ml Gey's solution, allowing only a few cells to be released. These were centrifuged (5 min at 200 g) and resuspended in 0.95% sodium citrate. They were incubated at room temperature for 20 min and then centrifuged again. After removing the supernatant, a fixatve reagent (75% methanol: 25% glacial acetic acid) was added, without disturbing the cell pellet. After 1 min the fixative was replaced by a fresh lot and the cells resuspended by flicking the tube. After a further 1-2 hrs fixation the cells had settled and the fixative was changed again. A final change of fixative was amade a few hr later, immediately before making the slides. This was done by first allowing 2 or 3 drops of the cell suspension to fall separately on a perfectly clean.

microscope slide. After these drops had spread out but just before drying was complete (at which time interference colour fringes could be seen) a few puffs of air were directed onto the slide from a rubber bulb. Spreading of the metaphase figures was more efficient if the final phase of drying was made more rapid in this way. A further 3 drops were then added and the process repeated until a sufficient density of cells was attained. The dry slide was viewed under a microscope with 20x phase objective in order to monitor the drying process. The slide was stained for 1 hour in 2% orcein in 45% acetic acid, washed briefly in 45% acetic acid and dried. Where it was necessary to make chromosome preparations from a thymus graft without exposing the whole animal to colcemid, the chopped graft was exposed for 30 min at 37°C to 4 mg/ml Colcemid in Gey's solution before dissociating the cells and proceeding as before.

2. Scoring of Mitotic figures.

In good preparations, where the chromosomes are well spread there was very little doubt as to the presence or absence of the T6 chromosome (see Fig.11). This chromosome is some 1/3 - 2/3 as long as the smallest of the other chromosomes, and is correspondingly reduced in thickness. In a normal cell, on the other hand there are several small pairs about equal in size. In poorer preparations, where two or more chromosomes were absent or partly superimposed it was not always possible to be certain that the T6 chromosome was absent. In a very few cells it was difficult to decide whether the T6

chromosome was present or absent, even among clearly visible chromosomes. In the absence of precise criteria for rejecting these "unknowns" they were scored with whichever group seemed closest. There was an unconscious tendency to reject as unscorable those cells without an obvious T6 chromosome. To avoid this, search was made for suitable mitotic figures using a X 20 objective, by which magnification the T6 chromosome was not readily distinguished. Poorly spread mitoses were then discarded without changing to high power, while those which were well spread, and appeared to have the full complement of chromosomes were examined under high power (X 45 or X 95 oil). Once the decision had been made, under low power, that a cell was scorable, and provided that it was not obviously deficient in chromosomes, it was always scored.

Stathmokinetic index.

This was estimated from the same fixed cell suspensions which were prepared for chromosome analysis. The cells were centrifuged down and resuspended in 45% acetic acid. This had the effect of dissociating most of the cells which still remained in clumps. Mitotic and non-mitotic cells were counted in a Fuchs-Rosenthal haemocytometer using X 20 phase objective without staining.

GVH Mitotic Test: Selective stimulation of mitosis in competent cells.

To make this method work to the best advantage it would be necessary to explore variations in a number of parameters. Only the most preliminary attempt has been made to do this here

since it was felt that even in its crudest form the test provided sufficient information to warrant it being used in experiments. The following are some of these parameters which have been examined cursorily:-

1. Strain of host mice. $F_1$ hybrids of CBA with C57BL were used. This should be one of the hybrids against which CBA reacts most strongly, since C57BL only share 1 H-2 antigen with CBA and have at least 2 not present in CBA (Snell, 1958). However, similar considerations would suggest that hybrids of CBA with ASW and DBA/1 might be equally good, and with A.CA possibly even better, since Wigzell (personal communication 1962) found CBA cells were more active against this strain than against C57BL, as measured by spleen assay.

2. Dose of irradiation. A dose of 900r was used throughout.

3. Age of recipient and route of injection. At first intraperitoneal injections in young mice (2-3 weeks) were used. Later, much higher mitotic rates were obtained by intravenous injection; and adult mice were used because they survived irradiation better.

4. Donor inoculum. The spleen is unsuitable as an inoculum because the haemopoietic cells present in it are likely to increase the proportions of non-specific mitoses in the GVH reaction; for this reason lymph nodes were always used.

5. Organ examined for mitoses. More mitoses were usually found in the spleen than in the lymph nodes. For this reason and because the spleen made better chromosome preparations, it was usually the only organ examined.

6. Time after cell transfer. The optimum time for maximum specifically-stimulated donor mitoses was chosen in accordance with the results of Experiment 3 (described below) to be 3-4 days after cell transfer.

7. Number of cells injected. Although 8 million lymph node cells were sufficient to induce a maximal stathmokinetic index (see experiment 3 below), in most experiments the whole lymph node complement of each donor mouse was injected.

## EXPERIMENTS

1. Tests of histocompatibility between CBA/T6T6 and CBA mice.

This was done both by skin grafting and by transfer of cells sensitised to BSA. In the second method the survival of the sensitised donor cells was detected by the adoptive secondary response that they gave after injecting the recipients with BSA. The details of the method are given in Part II. In the present experiment a period of 4 weeks elapsed between cell transfer and testing with BSA, so as to allow time for rejection to occur. The sensitised donor mice were all CBA/T6T6, and the recipients were either CBA/T6T6 or CBA. The same recipients were all grafted with CBA/T6T6 skin 4 weeks after the adoptive immunity test.

The antibody titres (Table 2) show that host mice accepted the donor cells for at least 4 weeks, since these were able to make antibody in a secondary response to BSA. Thus any histoincompatibility must have been exceedingly slight, and too small to affect the results of subsequent experiments involving

Table 2. Experiment demonstrating histocompatibility between CBA/T6T6 and CBA strains of mice. A secondary response has been elicited from preimmunised CBA/T6T6 spleen cells 4 weeks after transfer to CBA or CBA/T6T6 recipients.

Antigen binding capacity of sera obtained 20 and 30 days after injection of 100 μg BSA (μg/ml):

| CBA recipients | | CBA/T6T6 recipients | |
|---|---|---|---|
| 20 days | 30days | 20 days | 30days |
| 137 | 103 | - | 88 |
| 136 | 90 | 108 | 85 |
| 165 | 104 | 109 | 70 |
| 192 | 122 | 100 | 72 |
| 119 | 162 | 185 | 114 |
| 127 | 86 | - | 66 |
| 210 | 115 | 108 | 109 |
| | | 150 | 78 |

transfer of CBA/T6T6 lymphoid cells to CBA recipients. None of the skin grafts which were viable at 8 days after grafting (i.e. had "taken") showed any sign of rejection 4 months later when the mice were killed.

2. Mitotic response of irradiated and non-irradiated hybrid cells when subjected to immunological attack.

In a GVH reaction, not only the donor cells but the host cells are stimulated to proliferate. Although cell proliferation can be greatly depressed by irradiation, doses of 900 r are unlikely to abolish it completely. The first experiment shows however, that the contribution of mitoses by the irradiated hybrid host seldom exceeds 5% of the total.

Five irradiated (C57BL x CBA)$F_1$ hybrid mice were each injected with 20 million CBA/T6T6 lymph node cells intravenously. In chromosome preparations made 4 days later the following proportions of T6T6 mitoses were recorded: 47/50, 51/54, 30/31, 15/15, 25/25.

The second experiment was similar except that the donor cells were a mixture of equal numbers of (C57BL x CBA)$F_1$ hybrid and CBA/T6T6, and 100 millions of this mixture was injected into each recipient. The following proportions of T6T6 mitoses were recorded in the recipient spleens: 35/51, 4/18, 13/26, 14/28, 23/41 and 9/30.

The hybrid donor cells produced as many or more mitoses than did the parental strain cells. They cannot thus be compared with the incompetent moiety of these latter.

3. Increase in stathmokinetic index with time after cell transfer, and its relation to the cell dose.

(a) Adult hybrid and CBA mice were irradiated and each injected with 1 mouse-equivalent (about 50 millions) of pooled CBA lymph node cells. Groups of them were killed at intervals of 1 - 5 days after this and estimates of the stathmokinetic index made as described above. This index in the hybrid recipients rose rapidly to a plateau in 3 days, and thereafter showed little change (Fig. 1a). In the CBA recipients the index was considerably lower, being about 10% of that in the hybrids. There was an indication of a further rise after the 4th day. The optimum time after cell transfer for making chromosome preparations was therefore taken to be 3 - 4 days, when the ratio of specifically to non-specifically stimulated mitoses was greatest.

(b) The second experiment was identical except in that the time was kept constant while the cell dose was varied. A dose of 8 million lymph node cells was found to be sufficient to produce the maximal stathmokinetic index (Fig. 1b ~~16~~). Presumably, at doses higher than this, the number of host cells the donor cells were in the majority,, and therefore the index remained steady at a value characteristic of them donor cells.

4. Comparison of mitotic response by cells engaged in GVH reaction with that of non-reacting cells.

The overall mitotic response was estimated by the incorporation of the thymidine analogue IUdR (iodouridine-deoxyriboside). This was prepared so as to include about 1 mc/mg of iodine - 131,

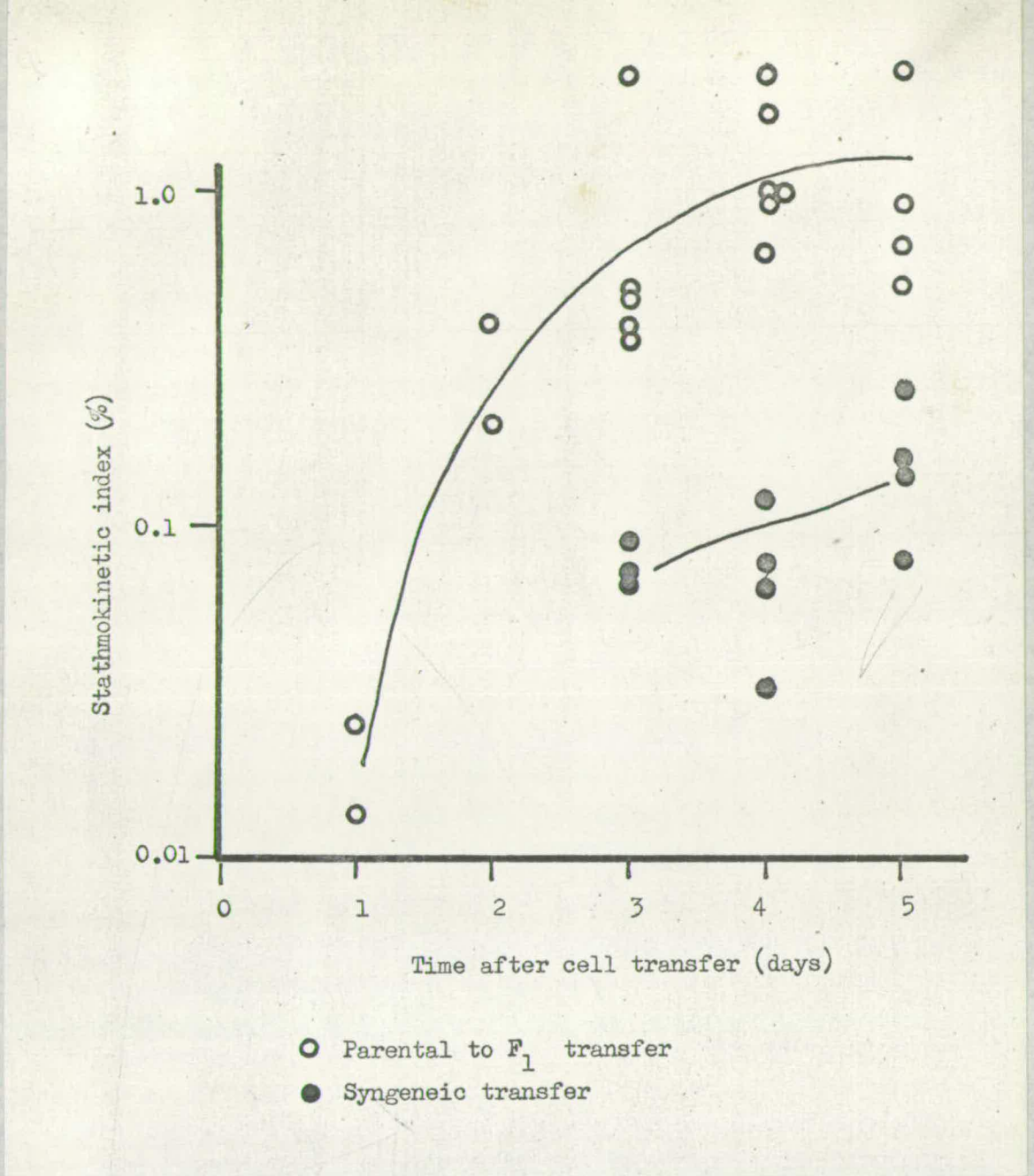


**Fig. 1. (a) Stathmokinetic index in spleen cells of hybrid and CBA irradiated mice after receiving CBA lymphnode cells. This diagram shows the development of the index with time after transfer of about 50 million cells.**

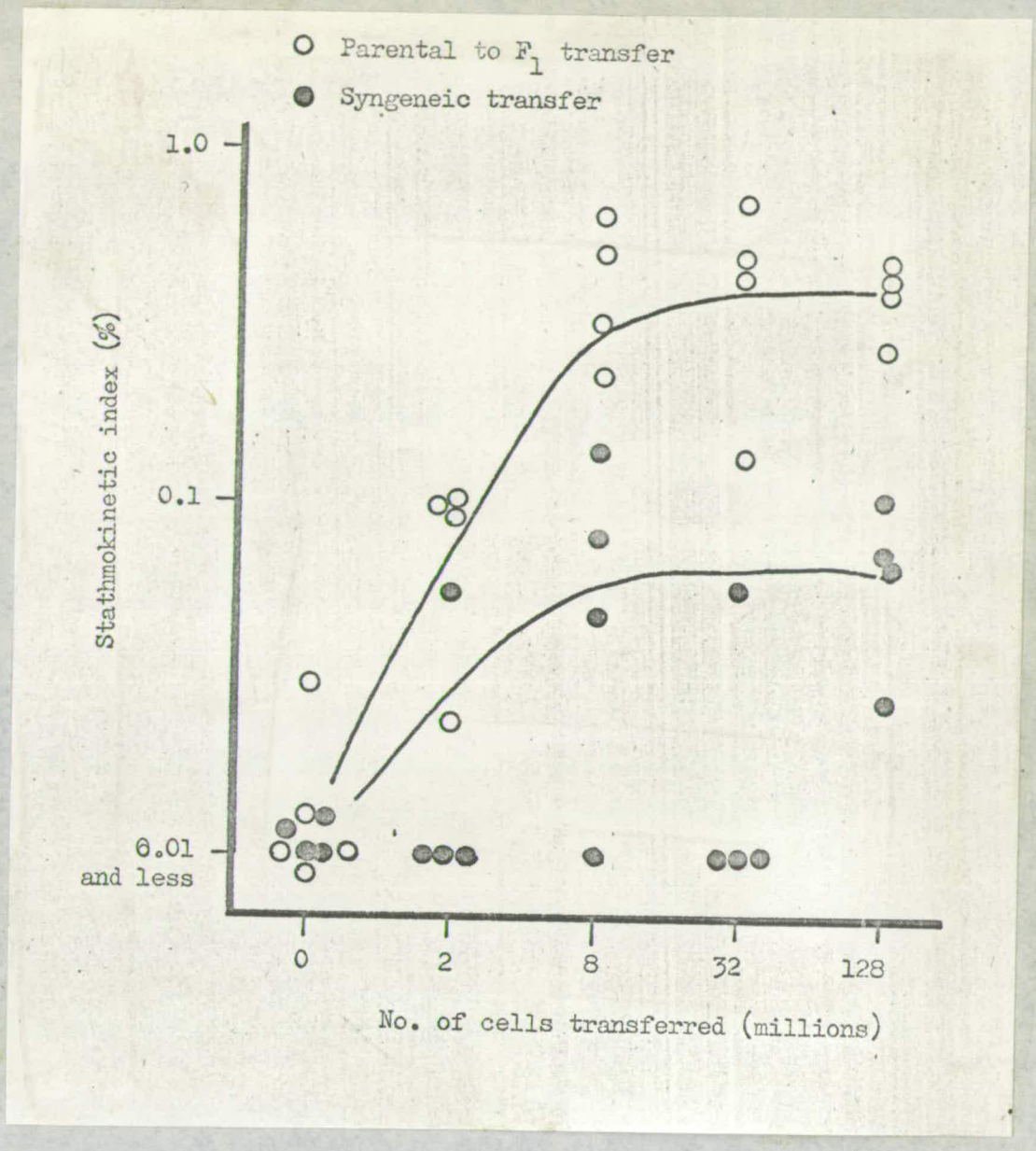


**Fig. 1(b).** This diagram is similar to Fig. 1(a), but shows the relation of stathmokinetic index to varying cell doses at 3 days after transfer.

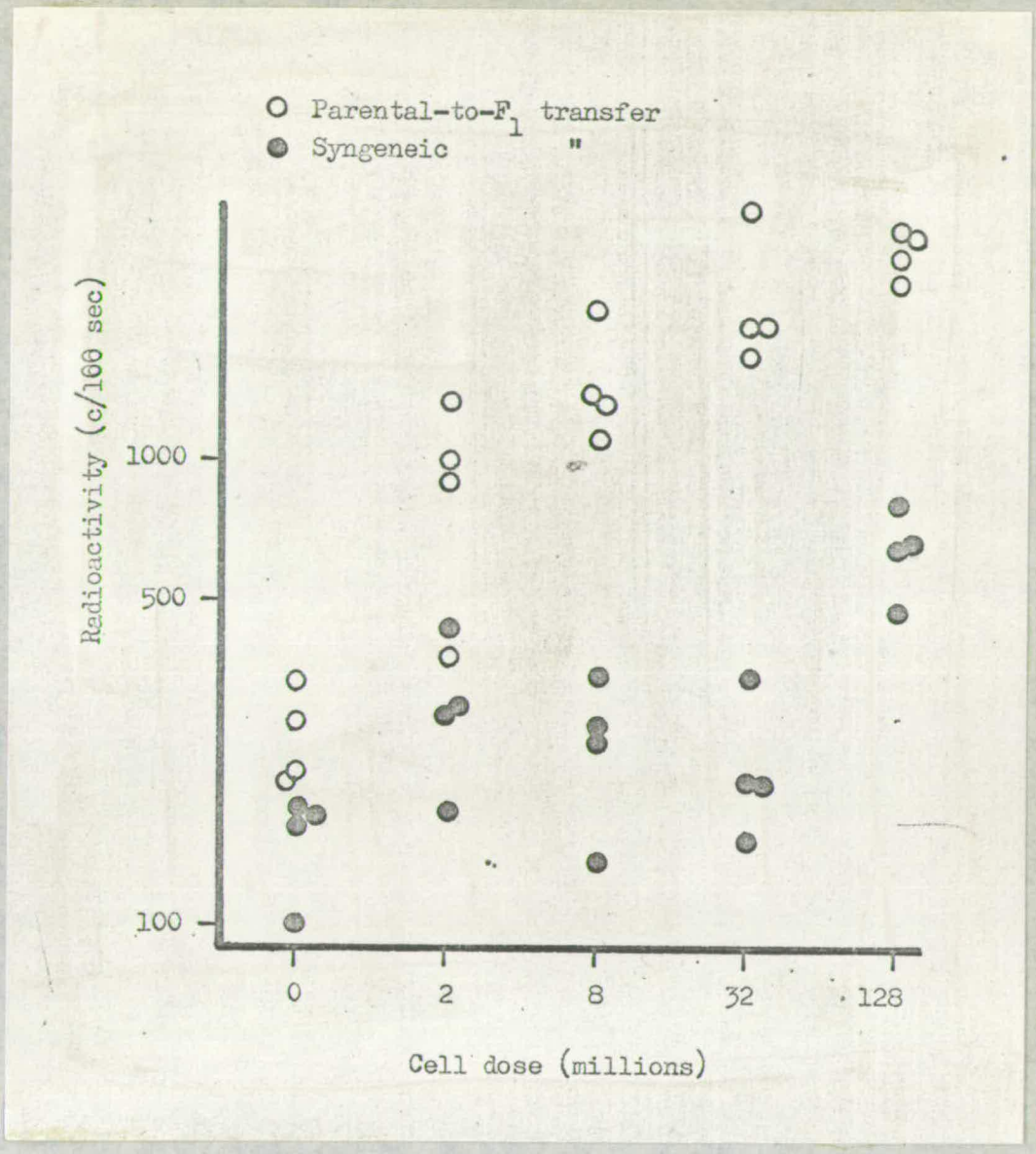


**Fig. 2.** IUdR uptake in spleens of irradiated hybrid and CBA mice three days after transfer of CBA lymph node cells. The diagram indicates a relation between cell dose and mitotic response.

by the method of Brownstone (1963). The mice were treated as in experiment 3b, but instead of being killed on the third day they were each injected with 2 mc of $^{131}$IUdR intraperitoneally. They were all killed 15 hours later, and the radioactivity of the spleens was determined. It can be seen in fig. 2 that even after transfer of 32 million syngeneic cells the IUdR-uptake was barely raised above the background. Only when 128 million syngeneic cells were transferred was there a significant uptake specific to the donor cells. Yet this result was equalled by the transfer of only 2 million cells in the parental-to-$F_1$ situation. Therefore it is unlikely that the proportion of donor mitoses unrelated to the GVH reaction would make up more than 2% of the total.

5. Equivalence in mitotic response of CBA/T6T6 and CBA cells.

There was no reason to suppose a priori that these two strains, which were shown to be sufficiently syngeneic to accept each other's skin grafts for at least 4 months, should differ in their immune responsiveness toward C57BL antigens. Nevertheless, it was considered that the point should be checked. Two pooled lymph node cell suspensions were made: one from CBA/T6T6 mice and one from CBA mice. These were mixed in various proportions and a total of 100 million cells injected into each irradiated hybrid recipient. In chromosome preparations the proportions between the two karyotypes were seen to reflect the proportions between the two types of cells injected (Fig. 3 ) with a slight preponderance of T6T6. The method of chromosome preparation in use at the time of this experiment resulted in

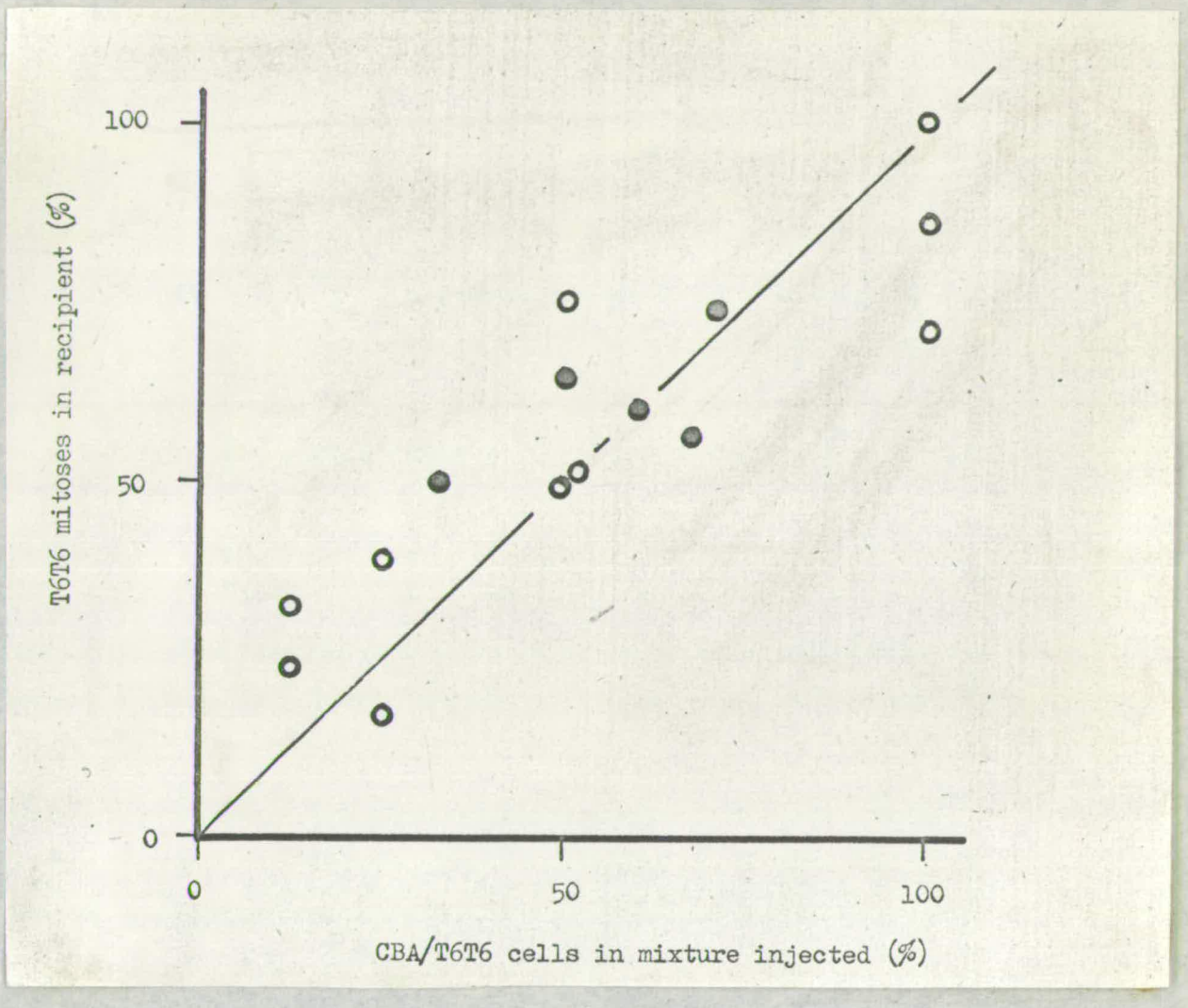


**Fig. 3. Mitotic competition experiments demonstrating the equivalence in GVH mitotic response of CBA/T6T6 and CBA lymph node cells. Symbols refer to two similar experiments.** each using a different pooled suspension containing equal numbers of the two types of cells.

very few of the mitoses (5 to 40 per slide) being sufficiently well spread to be scorable. The high variability in the results may thus be attributed to the small number scored.

6. Effect of pre-immunisation on mitotic potency.

(a) CBA/T6T6 and CBA mice were pre-immunised by being grafted with (C57BL x CBA)$F_1$ skin. An equal number were grafted with CBA skin and served as non-immunised controls. The allogeneic skin grafts were all rejected by 10 days after grafting. The cell transfer was made 22 days after grafting. Two pooled lymph node cell suspensions were made, containing equal numbers of cells from immunised and non-immunised mice. The only difference was that in one suspension the T6 marker was borne by the immunised cells, and in the other it was borne by the non-immunised cells. Aliguots of these suspensions, containing about 80 million cells, were injected intravenously to irradiated hybrid recipients as usual. Chromosome preparations were made at 2 days rather than 4 days after transfer. It was thought that, since the secondary response is usually more rapid than the primary, preparations made at 2 days would be likely to favour mitoses by the pre-immunised cells. In spite of this, the majority of mitoses were contributed by the non-immunised cells (Table 3).

(b) The second experiment was identical except in that the karyotype analyses were made at intervals from 1 to 4 days after cell transfer. Also, the interval between skin grafting and cell transfer was longer. No mitoses were found on the first day, but the results on subsequent days echo those obtained in the previous experiment (Table 3). It is concluded that the pre-immunised cells made the smaller mitotic response.

Table 3. Mitotic competition between equal numbers of pre-immunised and normal lymph node cells after transfer to irradiated hybrid recipients.

| Days after immunisation | Days after cell transfer | Karyotype of immunised cells: T6T6 T6⁻ | | | Karyotype of immunised cells: T6⁻ | | |
|---|---|---|---|---|---|---|---|
| | | T6T6 | T6⁻ | % Imm. | T6T6 | T6⁻ | % Imm. |
| 21 | 2 | 2 | 11 | 15 | 10 | 2 | 17 |
| | | 1 | 5 | 17 | 11 | 3 | 21 |
| | | 2 | 8 | 20 | 7 | 2 | 22 |
| | | 2 | 7 | 22 | 9 | 2 | 18 |
| | | 1 | 4 | 20 | | | |
| | | 1 | 5 | 17 | | | |
| | | Mean proportion from immunised donor = 19% | | | | | |
| | | T6T6 | T6⁻ | % Imm. | T6T6 | T6⁻ | % Imm. |
| 35 | 1 | 0 | 0 | - | 0 | 0 | - |
| | 2 | 9 | 22 | 32 | 17 | 8 | 32 |
| | 3 | 4 | 21 | 16 | 15 | 7 | 32 |
| | 4 | 7 | 15 | 32 | 18 | 12 | 40 |
| | | Mean proportion from immunised donor = 31% | | | | | |

## DISCUSSION

It is not known exactly what is the cellular basis of immune competence, but there are reasons to suppose that the lymphocyte is both necessary and sufficient for the initiation of immune reactions - at least to tissue antigens. These are mentioned in the discussion to Part II. There is also evidence that in GVH reactions the donor lymphocytes enlarge and divide rapidly. In the context of the mitotic test just described, then, some at least of the mitoses must stem from competent cells - indeed there is no reason to believe that any other type of cell undergoes much division during an immune reaction. However, the mitotic response of a cell suspension is not necessarily related to its immune potency. This was indicated in experiment 5, where pre-immunisation of parental-strain donors (which would presumably increase the immune potency as measured by splenomegaly reaction) actually depressed the mitotic potency. This may mean that the immunised cells, being in a higher state of differentiation, had lost some of the power to proliferate. At all events it indicates that caution should be used in the interpretation of mitotic responses. Nevertheless, in the absence of pre-immunisation, it is probable that mitotic response does represent a facet of immune potency, of which it can be taken as a quantitative measure within any one antigenic test system. The error which would be introduced by mitoses unrelated to the GVH reaction

was shown to be quite small. It might become important only if the immune potency of the cell suspension under test was very small, but is hardly likely to interfere with the conclusions drawn from the experiments in Part II. Ideally, it should be controlled in every experiment, by making a syngeneic transfer, but where this was attempted (e.g. experiment 13b) it was difficult to find enough mitoses to score an adequate number.

It would be an advantage to be able to measure in the same mouse the overall immune potency, as well as the contributions to this by the two karyotypes. One way would be to measure the stathmokinetic index on the suspension used for karyotype analysis. This has been attempted in one experiment (no. 13b), but it is not a good method since this index is not sensitive to changes in potency except in the lower ranges, where counting would be very tedious (see experiment 3b above). It would be possible to obtain a rough estimate of the number of dividing cells in the spleen by IUdR-uptake (experiment 4), but many uncertain factors, such as the effect of GVH on background uptake, would raise difficulties in the development of a precise method. A more elegant way would be to compare test and standard cell suspensions directly by "mitotic competition" within the same irradiated hybrid recipient, as was done in experiments 5 and 6. This ~~would have~~ has the great advantage of eliminating the effects of host variability. However, if the test animal is to contain two karyotypes (e.g. T6T6 and T6$^-$), as in the

experiments in Part II, then the standard animal must be of the third karyotype, T6. This adds to the labour of scoring and sets a more stringent requirement for quality in the chromosome preparations. It has not yet been examined extensively, but remains a potentially useful method.

## PART II: THE FUNCTION OF THE THYMUS IN THE DEVELOPMENT OF IMMUNOLOGICAL RESPONSIVENESS.

### INTRODUCTION.

As mentioned in the general introduction, there were good reasons for believing that thymectomy in early life might affect the development of immunological responsiveness. Many have realised this and several have put the idea to experimental test. There is by now much published work on the effects of thymectomy on immunological responsiveness, and this will be discussed later in conjunction with my own results.

The general conclusion that the thymus is necessary in early life for the proper development of lymphoid tissue and of immune responsiveness is beyond question, but the means by which it acts is less clear. The development of lymphoid tissue must include two essential processes: the first is maturation of incompetent cells to competent cells, and the second is proliferation. Maturation may occur before, during or after proliferation. It seems most likely that it is on these processes that the thymus exerts its influence; but it is formally possible that the thymus should influence the development of something, other than the competent cells themselves, which is necessary for the proper function of these cells. In the first series of experiments (nos. 7 - 10), the effect of neonatal thymectomy on immunological responsiveness is examined using various test systems. The second series (nos. 11 - 13) provide evidence that the influence of the thymus is in fact

exerted on the lymphoid cells themselves.

The action of the thymus upon the development of lymphoid cells may be envisaged to accord with three principal schemes, which are outlined in Fig. 4. The essential developmental changes are therein represented as the division and maturation of a hypothetical stem cell to form two competent cells, and it is assumed for the present that division and maturation occur together. In the first scheme, the "germinal" hypothesis, the thymus is seen as containing all the stem cells necessary for complete development of lymphoid tissue; and during this process these cells would mature and leave the thymus to colonise the rest of the body. In the second, or "humoral" hypothesis, the thymus would not contain lymphoid cells, but would secrete a hormone essential for lymphopoiesis elsewhere in the body. In the third, which might be termed the "finishing school" hypothesis, (after Burnet, 1963), the stem cells would arise from somewhere outside the thymus and pass through this organ during their maturation towards competent cells. Some evidence for the occurence of the "finishing school" mechanism in post-natal life is presented in experiments 13a and b.

It has been held by many authors that in embryonic life the first lymphocytes to appear in the thymus arise by differentiation from the thymic epithelium. If these lymphocytes were then seeded out to the rest of the body it would accord with the germinal hypothesis. Experiments 14 and 15 suggest, however, that the finishing school mechanism can occur in the embryo, while the germinal mechanism, if it occurs, is probably of little importance.

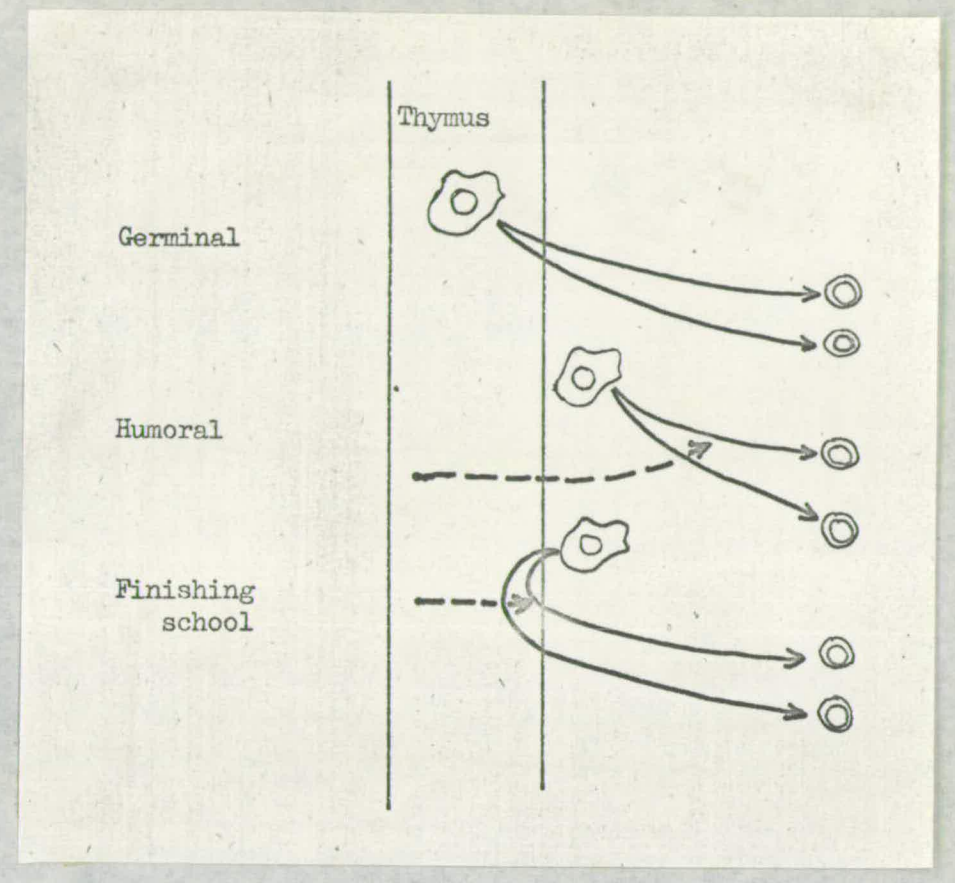


Fig.4. Three hypotheses concerning the mode of action of the thymus in bringing about development of the lymphoid system. In each case a stem cell is seen as dividing and maturing to form two competent cells. Dotted arrows indicate humoral or other influences specific to the thymus.

There is evidence, discussed later, that the bone marrow is a rich source of lymphoid stem cells, so defined by their capacity to enter and repopulate the thymus. Since marrow also contains haemopoietic stem cells, the question arises whether cells exist which combine these potentialities. Experiments 15 and 16 suggest that they do.

One consequence of both the germinal and finishing school hypotheses is that thymus cells themselves must be potentially competent. As mentioned in the general introduction, it is possible that a high proportion of them might already have undergone the thymus-dependent step in their maturation and should therefore be capable of becoming competent after release from the thymus. In experiments 17 - 21 some responses of dissociated thymus cells are examined, and it is shown that in some situations they can become highly ~~competent~~ potent, although their ~~competence~~ potency immediately after release from the thymus was invariably found to be low.

# MATERIALS AND METHODS.

## Animals.

Mice of the strains CBA, CBA/T6T6, and $F_1$ hybrids of CBA with C57BL were used.

## Antigens and immunisation.

Bovine serum albumin (BSA), as used for immunisation and paralysis, was Fraction V prepared by the technique of Cohn et al (1946). The BSA used for the titration of antibody by Farr's method had been further purified by recrystallization.

Bovine gamma globulin (BGG) was Fraction II from the Cohn separation. All proteins were obtained from Armour and Co.

Freund's adjuvant ("Freunds") was prepared and injected as described by Dresser (1960).

Alum precipitation of proteins was carried out according to the method of Proom (1943). The alum precipitates of BSA or BGG alone have poor immunising ability in mice, but when mixed with Bordetella pertussis vaccine (Burroughs Wellcome) their immunogenicity is much enhanced (D.W.Dresser, personal communication, 1963). Equal quantities of alum precipitate and B. pertussis (containing $2 \times 10^{10}$ organisms per ml.) were mixed and stored with 1:10,000 merthiolate at 2°C. The preparations are hereafter referred to as BSA-AP (or BGG-AP). To immunise, 0.2 ml. was injected intraperitoneally.

Salmonella typhi. H. antigen (STH) was supplied by Burroughs Wellcome, Ltd. To immunise, 0.1 ml was injected intraperitoneally.

Test Bleeding.

Mice were bled 6 or 7 days after immunisation with STH or SRBC, and either once, at 28 days, or twice, at 20 and 40 days after immunisation with BSA. They were bled from the ventral tail artery.

Adoptive secondary response to BSA.

When lymphoid cells from a mouse at the height of antibody production against BSA are transferred to a syngeneic, non-immune mouse, their production of antibody almost ceases. Yet the recipient animal becomes responsive to subsequent injections of BSA (Mitchison, 1962). No adjuvant is necessary to elicit this secondary response, and since the primary response of mice to BSA without adjuvant is negligible, the adoptive secondary response is very largely a measure of the state of immunisation of the donor cells. To elicit such a secondary response the recipients were injected intraperitoneally with 100 µg of protein dissolved in saline, either immediately, or 1 - 28 days later. The response obtained was found to be equally vigorous independent of time within these limits. They were bled 10 and 20 days after this injection.

Trace labelling the proteins with radioactive iodine.

Iodine - 131 was attached to the protein by McFarlane's iodine chloride method (1958). The labelling ratio varied from about 1 - 6 atoms of iodine per molecule of BSA for use in Farr's technique; or 0.3 - 1 per molecule of BGG for use in the antigen-elimination technique described in Part III.

Antibody titrations.

1. Sheep red cells.

Doubling dilutions of the serum were made in buffered saline in plastic trays so as to leave 0.25 ml of diluted serum in each chamber. Then 0.1 ml of 1% sheep red cells was added, and the trays shaken and left overnight at 4°C. The endpoint was taken as the highest dilution in which the pellet was distinctly larger than that in the control chamber containing serum from non-immunised mice at the same dilution.

2. Salmonella typhi.H.

This titration was the same as that described above for sheep red cells, except that 3/8" diameter glass tubes were used instead of plastic trays. The volume of diluted serum was 0.1 ml and to this was added 0.1 ml of STH suspension.

3. Bovine serum albumin.

The method used was basically that of Farr (1958). In brief: Radioiodine-labelled antigen (BSA in this case) is added to the antiserum and time allowed for the formation of antigen-antibody complexes. Then, by adding ammonium sulphate, the labelled antigen bound in the complexes is precipitated (along with all globulins) while the uncomplexed antigen is left in solution. From the proportion of the total radioactivity precipitated the "antigen-binding-capacity" (ABC) of the serum can be obtained. As adapted by Dr. N.A.Mitchison, and used here, the method was as follows:

Four glass tubes (3/8" x 2") were set out for each sample of antiserum to be tested. In the first tube 0.1 ml of the

antiserum was diluted either 6 times or 11 times (depending on the antibody titre anticipated) by adding either 0.5 ml or 1.0ml of borate buffered saline (Farr, 1958) from an automatic device. To the third and fourth tubes was added 0.25 ml and 0.2 ml respectively of borate buffered saline containing 10% normal rabbit serum (10% NRS). Three sets of control tubes were set up in quadruplicate. The first and second sets contained 0.25 ml of 10% NRS, and the third 0.25 ml of normal mouse serum at the same concentration as the lowest dilution of mouse antiserum used. Using constriction micropipettes for the further dilutions, 0.05 ml of the first dilution was mixed with the 10% NRS in the third tube and the same volume carried over from the third to the fourth tube. Into the empty second tube was put 0.25 ml of the original dilution. Prepared in this way the dilution ratio should be 6 between the second and third tubes, and 5 between the third and fourth, while the volume of fluid should be 0.25 ml in each of the three. The contents remaining in the first tube were stored until the titration had been completed, in case it was necessary to repeat any of it. An equal volume (0.25 ml) of $^{131}I$ -BSA (2 µg/ml in 1% NRS - borate) was then added to each tube and mixed by shaking. After 24-48 hrs. at 4°C the first set of control tubes (containing 10% NRS) received 0.5 ml 10% trichloracetic acid, while all other tubes received 0.5 ml saturated ammonium sulphate. Immediately after this addition each tube was shaken on a vortex mixer. They were kept at 4°C for 30 mins., and then centrifuged at the same temperature for 30 mins. at 1800g. The supernatant was poured

off and the tubes inverted on absorbent paper to drain. The radioactivity of the precipitates was read in a scintillation counter. The total protein-bound radioactivity (TCA) was read in the tubes receiving trichloracetic acid, while the radioactivity carried down non-specifically with the ammonium sulphate-precipitated mouse or rabbit serum proteins (SAS) was read in the other control tubes. From the radioactivity reading of each tube containing antiserum, the percentage of the available antigen specifically bound by antibody was obtained as follows:-

$$\% \text{ binding} = \frac{\text{Reading} - \text{SAS}}{\text{TCA} - \text{SAS}} \times 100$$

The relation between the percentage of antigen bound and the ABC of the diluted antiserum is not linear, but follows a curve such as Fig. 5. This was constructed by titrating a range of dilutions made from 3 pools of antiserum obtained at different times after immunisation. The three curves were superimposed by graphical means at the point where they cut the ordinate value of 33% precipitation. The arbitrary units of concentration were then converted to units of ABC in accordance with the definition of the unit of ABC as 3 times the concentration of antibody which binds 33% of the available antigen (Farr, 1958).

Any value of percentage binding can be converted to ABC with the use of this curve, and the ABC of the undiluted antiserum obtained by multiplying by the dilution of the antiserum. In practise, in most experiments, the assumption was made that the curve was linear between 10% and 35% precipitation. This

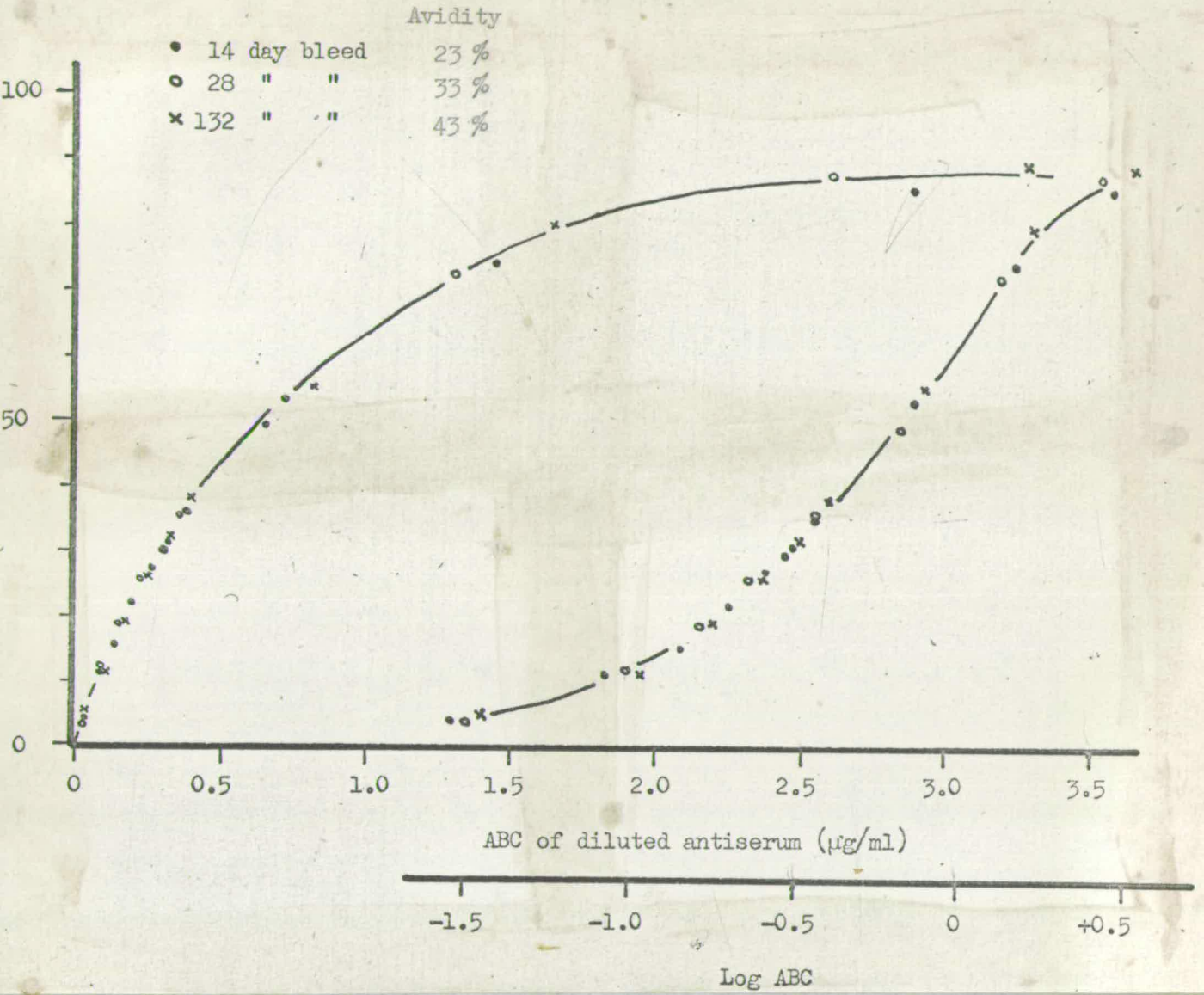


**Fig. 5.** This diagram illustrates the relation between the percentage precipitation of antigen in Farr's method and the concetration of antiserum (left hand curve) or log concentration (right hand curve). It demonstrates that this relation remains the same for sera obtaines at different times after primary immunisation. It also shows that the relation is approximately linear up to 35% precipitation, and logarithmic from 35% to 75%.

assumption might involve errors of up to 10% but this disadvantage was outweighed by the speed and freedom from human error of calculation by machine, rather than working from the graph.

The figures for ABC thus obtained were fed into London University's "Mercury" computer to obtain geometric means and standard deviations. For this purpose, all figures for ABC were treated as positive, and those which fell below 0.01 µg/ml were corrected to this figure. The conditions of the titration were such that figures less than 0.1 µg/ml were of borderline significance, but in the experiments which follow, no conclusions are based on an absolute distinction between presence or absence of antibody, and little weight is attached to differences between titres falling below 1.0 µg/ml.

The mean avidity of antibody from an immunised animal increases with the time after immunisation. It was possible that the shape of the dilution curve might differ for antisera of different avidity, in which case the determination of ABC would require a separate curve for each type of antiserum. The avidities of the 3 pools of antiserum used to construct the curve were determined by titrating the antisera against antigen concentrations of 2.0 µg/ml and 0.2 µg/ml and expressed as:

$$\text{Avidity} = \frac{\text{ABC at 0.2 µg/ml}}{\text{ABC at 2.0 µg/ml}} \times 100 \quad \text{(Farr,1958)}$$

It is evident that although the avidity at 14 days after immunisation was distinctly less than at 132 days, the shapes of the curves do not differ seriously (Fig. 5). In most of the

subsequent experiments avidity determinations have not been done. It was thought justifiable to assume that where the experimental and control mice were immunised by the same means, and bled at the same time after immunisation, the avidity would be the same for antisera from each group - especially as it was not influenced by thymectomy (see experiment 10 below).

Thymectomy.

1. Neonatal mice.

Anaesthesia was achieved by hypothermia. Three glass tubes (1.5 cm x 7.0 cm) were set upright in a dish containing water and crushed ice. The mice were put in their tubes in sequence so that during thymectomy of one, two others could be cooling. Each was cooled for 5 - 10 mins. by which time breathing and heartbeat had usually stopped. When using a head-light it was found that the heat from this terminated anaesthesia too quickly. Therefore a heat filter was put in front of the bulb. The mice were laid on a plastic board so that a raised portion of the board was situated under the thorax and served to make the thymus more accessible. They were held to the board by means of strips of adhesive plaster. A midline incision was made through the sternum (or just to the side of it) and passing as far as the third intercostal space. For mice less than one day old a piece of sternum was removed with the use of a 1 mm glaucoma punch (J.Dixey and Co. Wigmore St., London, W.1 ). The two lobes of the thymus were removed sucessively by suction applied at the caudal end of the lobe, with gentle traction, using a glass pipette connected by a rubber tube to a mouthpiece. The pipettes

were made from a 6 mm or 8 mm glass tubing drawn out to the required diameter (about 0.8 - 1 mm internal diameter for new-born mice up to 3 mm for 3 week old mice.) A constriction was made about 4 - 7 mm from the tip and at the same time the end was bent at an angle. The constriction made it possible to retain each thymus lobe and then blow it out into a dish of Gey's solution, where it could be kept for subsequent implantation, if required. After the thymus was removed the skin was sutured with one stitch of no. 00 multistrand nylon, and the wound covered with collodion. The mice were laid on an electrically-warmed pad until normal movement was restored. They were then returned to their mother.

2. Older mice.

By the time the hair has grown, at about 14 days old, mice were found to be less tolerant of hypothermia induced with iced water. To anaesthetise these older mice, they were injected with 0.01 ml/g body weight of a 2% solution of Avertin (Winthrop laboratories, New York) in saline with 10% ethanol. The mode of removing the thymus was the same as that used for neonatal mice, except that the chest incision was only continued as far as the second intercostal space. By this means it was usually possible to avoid breaking into the pleural cavity. If air did enter the thorax it was often possible to save the mouse by first widening the hole in the pleura with forceps, and then squeezing out the air. Pneumothorax was no problem in the neonatal mice.

At the end of any experiment, thymectomised mice were always examined for thymus remnants. A few mice in some of the

early experiments were found to be incompletely thymectomised. Later, the procedure was adopted of discarding any mouse of which the thymus was broken during thymectomy. When this was done, no thymus remnants were found at autopsy.

Thymectomy with irradiation.

For many purposes it was useful to have mice whose responsiveness to antigen would remain permanently depressed. Neonatal thymectomy was unsatisfactory because of the high mortality, first from maternal neglect, and then from wasting disease (Parrott and East, 1962). The finding, which is discussed in Part III, that mice thymectomised in adult life did not recover responsiveness after irradiation, offered a much more practical method. This has been used extensively in the following experiments. It was as follows:

Mice of both sexes (but predominantly females) were thymectomised at 2-3 months. Irradiation was done at least 14 days after thymectomy, and sometimes as long as 2 months after. There was never any indication that variation within these limits made any difference to the result. The dose was usually 900 r, but in one experiment it was 600 r. Mice receiving the higher dose were injected immediately afterwards with about 7 million syngeneic bone marrow cells as protection against too early death. As described later, there was no tendency for responsiveness to recover spontaneously in these mice, although it could be restored at any time by inoculation of lymphoid tissues.

Implantation of thymus grafts.

These were put under the kidney capsule. The recipient mouse was first anaesthetised with Avertin; then the left kidney was brought out through a flank incision, and the implant ( 1-3 day old thymus) inserted under the capsule by means of a trochar The adult thymus was too large to be pushed under the kidney capsule by this means. It was implanted as follows: a number of adult thymuses (usually from 6-8 week old mice) equal to the number of recipients, were chopped finely with scissors. The resulting slurry was drawn up into a 1 ml tuberculin syringe. This was then fitted with a no. 16 needle with the neck bored out to the same diameter as the shaft, and used to inject an aliquot of thymus slurry under the kidney capsule.

## EXPERIMENTS AND RESULTS.

### Effect of thymectomy on immunological responsiveness.

7. Thymectomy at 7 - 14 days old.

Fifteen mice were thymectomised and 14 littermates sham-operated. Apart from a few deaths shortly after operation, all mice remained healthy throughout the 6 months of the experimental period. Some of the mice were challenged with BSA Freunds at 4 months of age. The resulting titres of antibody at 20 and 40 days after challenge can be seen in Table 4. The thymectomised group produced only about 1/10 as much antibody as the sham-operated controls. As there was little difference between the responses of males and females, they have not been distinguished in the table. The same mice were then tested for their response

to other antigens. Half of them were challenged with STH and then all were grafted with skin from a C57BL donor. The effect of thymectomy on antibody production to STH was comparable to the effect on anti-BSA production (Table 5), while the survival of C57BL skin homo-grafts was somewhat prolonged (Table 6).

8. Thymectomy at 1-7 days old.

This time a number of the thymectomised group did not grow so well as the controls, and three died before they were 3 months old, at which time they were challenged with BSA Freunds. The titres of antibody at 20 and 40 days after challenge can be seen in Table 4. The impairment of antibody production was more marked than in the previous experiment. It was greater in the males than in the females. Thymus fragments were discovered in a few of the mice. These mice produced antibody titres intermediate between the thymectomised and control mice.

9. Thymectomy at less than 2 days old.

About half of the thymectomised group were obviously cachectic when weaned at 5 weeks old. Five died between then and antigenic challenge at 10 weeks. BSA was not used in these mice because it appeared probable that many more of them would have died before they reached an age at which an adequate response to BSA can be obtained. Instead, the males were challenged with SRBC, and the females with STH. Among the males the responses to SRBC of the thymectomised group were much lower than the controls (Table 5). The impairment may have been even greater than that seen in experiment 8, but

Table 4. Anti-BSA antibody production in mice thymectomised (Tx) or sham-operated (Sh) at 1 - 7 days old (a), or 7 - 14 days old (b).

| | | No. in group. | Mean antigen binding capacity (μg/ml) at: 20 days | 40 days |
|---|---|---|---|---|
| Males (a) | Tx | 7 | 0.92 | 5.95 |
| | Partly Tx | 4 | 11.9 | 71.7 |
| | Sh | 8 | 17.7 | 150.0 |
| Females | Tx | 5 | 5.88 | 22.8 |
| | Partly Tx | 3 | 6.72 | 56.0 |
| | Sh | 8 | 36.7 | 234.0 |
| Both sexes (b) | Tx | 9 | 2.38 | 7.08 |
| | Sh | 7 | 16.2 | 102.0 |

Table 5. Anti- sheep red cell (SRBC) and anti-*Salmonella typhi* H (STH) agglutinin production by neonatally-thymectomised (Tx) and sham-operated (Sh) mice.

| Antigen. | Age at thymectomy (days) | Age at challenge (weeks) | | No. of mice producing serum agglutinating to the following $\log_2$ titre: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | <5 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| SRBC | 0-2 | 10 | Tx | 7 | 1 | 2 | - | 1 | - | - | - | - | - | - |
| | | | Sh | - | - | 1 | 1 | 5 | 2 | - | - | - | - | - |
| | 0-2 | 10 | Tx | - | 1 | 3 | 4 | 1 | - | - | - | - | - | - |
| | | | Sh | 1 | 2 | 4 | 3 | 1 | - | - | - | - | - | - |
| STH | 3-5 | 26 | Tx | - | - | - | - | - | 1 | 2 | 1 | 2 | - | - |
| | | | Sh | - | - | - | - | - | - | - | - | 1 | 1 | 4 |
| | 7-14 | 22 | Tx | - | - | - | 1 | 2 | 1 | 2 | - | - | - | - |
| | | | Sh | - | - | - | - | - | - | 1 | 1 | 3 | - | 1 |

Table 6. Effect of thymectomy at 7 - 14 days old on the duration of survival of C57BL skin allografts.

| | Day after grafting of complete rejection: |
|---|---|
| Thymectomised | 9, 9, 9, 9, 11, 12, 12, 13, 15, 15, 17, 17. |
| Sham-operated | 9, 9, 9, 9, 9, 9, 9, 9, |

Table 7. Avidity of anti-BSA antisera from normal and thymectomised mice. Thymectomy was at 1 - 7 days, and immunisation at 4 months of age. The mice were bled 40 days after immunisation.

| | Antigen binding capacity (μg/ml) when Tested against the following concentrations of BSA: | | Avidity |
|---|---|---|---|
| | 1.0 μg/ml | 0.1 μg/ml | (%) |
| Thymectomised | 4 | 2.3 | * 58 |
| | 14 | 1.7 | * 24 |
| | 18 | 5.4 | 30 |
| | 19 | 4.6 | 24 |
| | 40 | 12.0 | 27 |
| | 33 | 8.9 | 27 |
| | 95 | 21.0 | 22 |
| Not operated | 28 | 7.0 | 25 |
| | 140 | 37.0 | 24 |
| | 110 | 27.0 | 24 |
| | 140 | 41.0 | 24 |
| | 230 | 53.0 | 23 |
| | 240 | 55.0 | 23 |
| | 280 | 64.0 | 23 |
| | 300 | 72.0 | 24 |

*These figures are not reliable because of the low titre at 0.1 μg antigen/ml.

unfortunately, owing to the small volume of serum obtained from the cachectic mice, the titration was not carried to dilutions lower than 1/32. Among the females, the response to STH was not impaired at all.

10. Avidity of anti-BSA antibody from thymectomised mice.

Antisera from thymectomised and sham-operated mice were selected to include high and low antigen binding capacities in each group. Avidity was determined as described in the section on materials and methods, except that the concentrations of $^{131}$I-BSA were 1.0 µg/ml and 0.1 µg/ml instead of 2.0 µg/ml and 0.2 µg/ml. No significant difference in avidity was found between the two groups (Table 7).

Restoration of immunological responsiveness to thymectomised mice; the origins of the competent cells.

It was evident from the effect of neonatal thymectomy noted in the previous section, and its relative ineffectiveness if performed in adult life, that the influence of the thymus is primarily developmental, and that it has little or no immediate action on the immune functions of the adult animal. The object of the first two experiments was to determine whether the immunological defect of the neonatally-thymectomised mouse lay in the lymphoid cells themselves, or in the environment in which they had to function. Experiment 11 compares the ability of normal lymphoid cells to undertake a secondary response in neonatally-thymectomised recipients, with the ability of

lymphoid cells from neonatally-thymectomised animals to undertake such a response in non-thymectomised recipients. In an analogous experiment (no.12) it is shown that primary responsiveness can be restored to neonatally-thymectomised mice by normal lymph node cells, and also by thymus grafts or dissociated thymus cells. In experiment 13 the GVH mitotic test which was described in Part I is employed to trace the origin of the competent cells in these restored mice.

11. Transfer of secondary responsiveness.

The mice used were those mentioned in experiment 7, which had been thymectomised or sham-operated at 7-14 days old, and had already received various antigenic challenges. In addition a number of normal mice were immunised with BSA Freunds. The mice from the operated group which had been injected with BSA Freunds were killed, and individual cell suspensions made from their spleens. These were injected into non-immunised recipients. Those mice among the operated group which had not received BSA Freunds served as recipients for a pooled spleen cell suspension made from an equal number of immunised normal donors. All the recipient mice were then injected with 0.1 mg BSA and bled 10 and 20 days later. The results are shown in Table 8. It is evident that the spleen cells from neonatally-thymectomised mice were much inferior to normal spleen cells in their capacity to transfer secondary responsiveness to BSA. The thymectomised recipients on the other hand were quite capable of supporting a secondary response by cells from a normal animal. However, the responses in the thymectomised recipients were slightly, though

Table 8. Adoptive secondary response to BSA. This experiment shows that neonatally-thymectomised (Tx) mice are capable of supporting and adoptive secondary response by cells from normal immunised donors (a), but that cells from immunised thymectomised donors are deficient in their ability to transfer this response to sham-operated (Sh) recipients (b).

| | Cell transfer | | | | Mean antigen binding capacity (μg/ml) at: | |
|---|---|---|---|---|---|---|
| | Immunised donors | | Non-immunised recipients | No. in group | 10 days | 20 days |
| (a) | Normal | → | Tx | 6 | 13.8 | 11.2 |
| | Normal | → | Sh | 7 | 20.2 | 17.4 |
| (b) | Tx | → | Normal | 9 | 0.93 | 0.72 |
| | Sh | → | Normal | 7 | 36.1 | 22.2 |

not significantly lower than in the sham-operated recipients.

12. Restoration of responsiveness to BSA by lymph node cells, thymus cells and thymus grafts.

Thirty male CBA mice were thymectomised and eighteen sham-operated at 2-3 days old. The same seven weeks later were divided into groups of six. Five of these groups (I-V), consisted of thymectomised, and three (VI-VIII) of sham-operated mice. They were treated with solid grafts or cell suspensions of lymphoid tissues as detailed in Fig. 6. As the same mice were used later in experiment 13, the general plan of the 2 experiments has been laid out in Fig. 7. The donors were of both sexes and were aged either 2-3 days ("neonatal") or 2-3 months ("adult"). Three of the transfers to each group were taken from CBA/T6T6 and the other three from CBA mice. After a further 7 weeks, all the mice were challenged with BSA Freunds and then bled 35 days later. The anti-BSA antibody titres are shown in Fig. 6, from which it will be seen that both dissociated lymph node and dissociated thymus cells were effective in restoring responsiveness. Neonatal thymus grafts were effective, but adult thymus grafts were not. Neither lymph node cells nor neonatal thymus grafts significantly altered the responsiveness of sham-operated mice.

13. Restoration of GVH responsiveness by lymph node cells, thymus cells and thymus grafts; the origin of the competent cells.

(a) The mice used were those which had already been challenged with BSA in experiment 12. The three mice in each group which had received T6-marked inocula were subjected to GVH

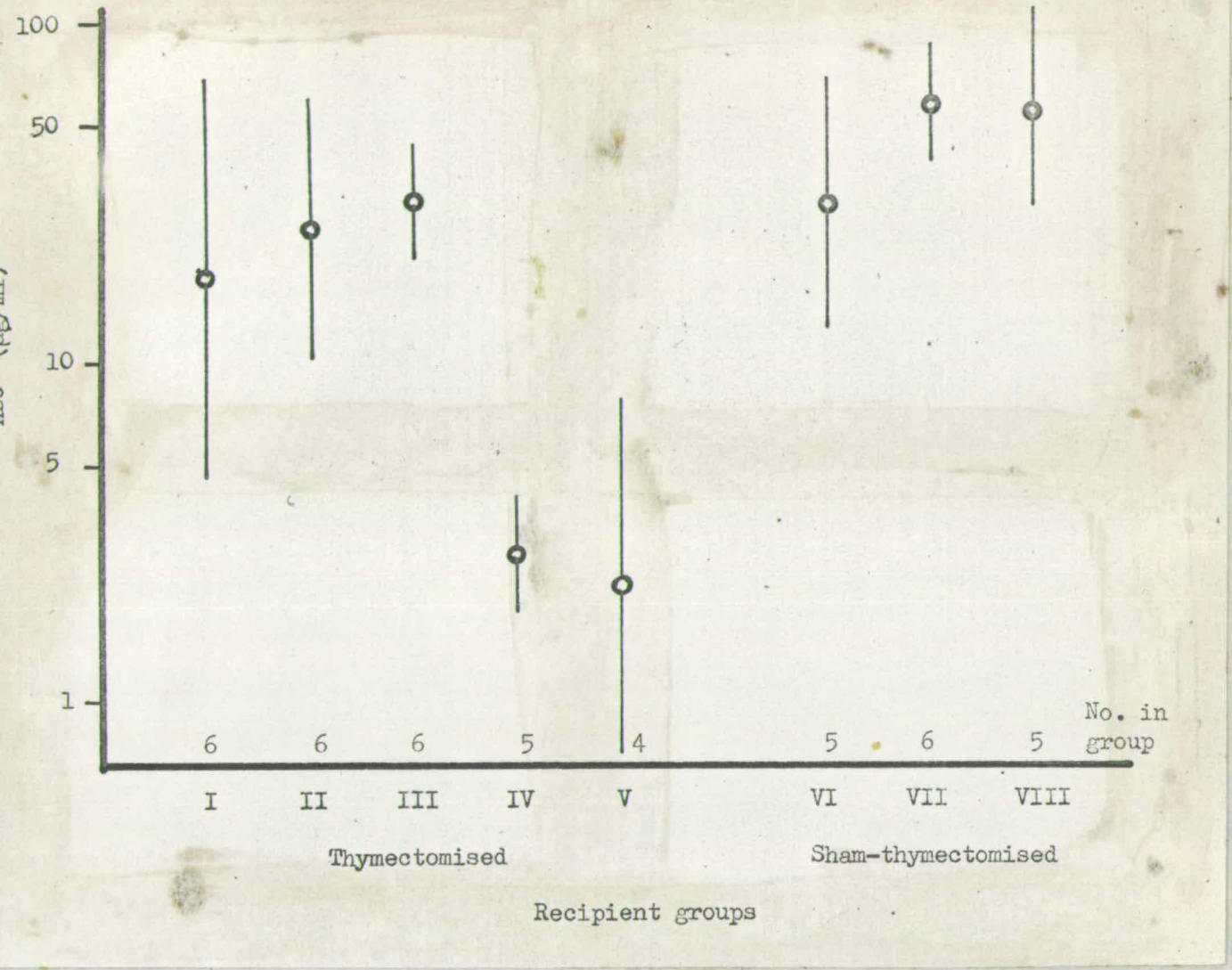


**Fig. 6.** Restoration of responsiveness to BSA in neonatally-thymectomised mice by transfer of various lymphoid tissues:

| | Recipient Group | Transfer |
|---|---|---|
| Thymectomised | I | 50 million adult thymus cells, i.p. |
| | II | 50 " " lymph node cells, i.p. |
| | III | 1 neonatal thymus graft. |
| | IV | approx. 1 adult " " |
| | V | No transfer. |
| Sham-operated | VI | 50 million lymph node cells, i.p. |
| | VII | 1 neonatal thymus graft. |
| | VIII | No transfer. |

Mean titres of antibody for each group are shown with standard deviations.

Further experimental details can be found in Fig.7.

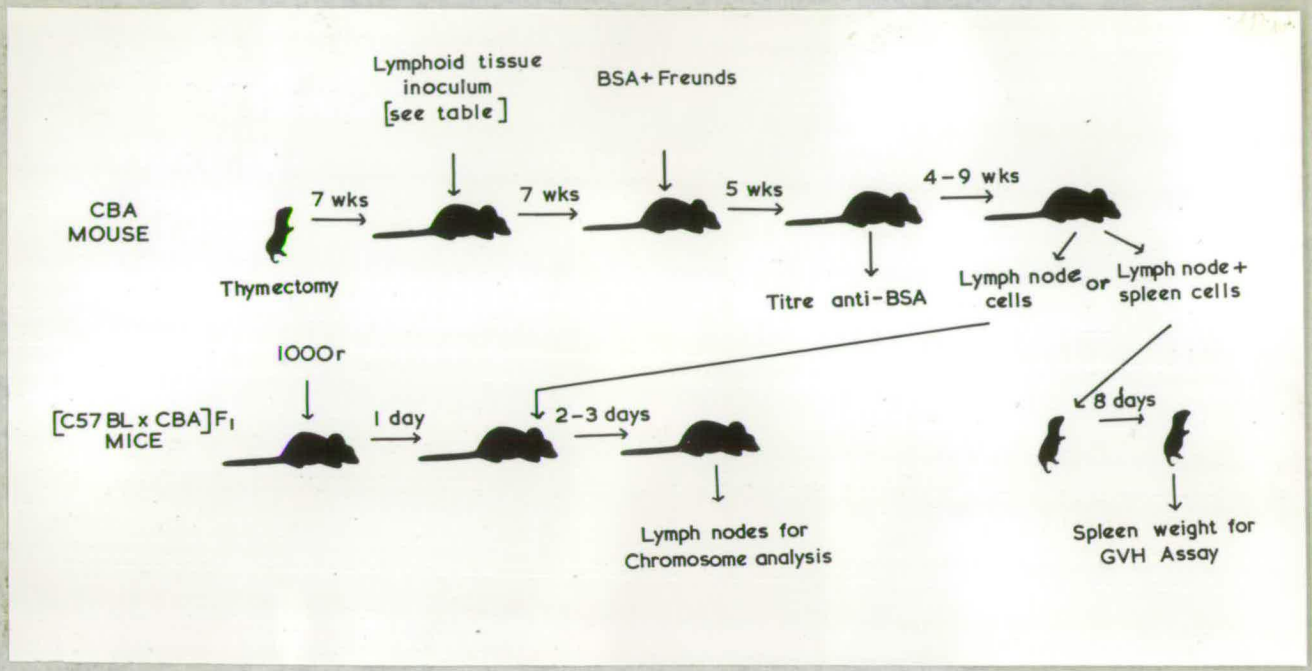


Fig. 7. This diagram illustrates the plan of experiments 12 and 13(a). The lymphoid tissue inocula and anti-BSA antibody titres are detailed in Fig. 6, while results of GVH assays and karyotype analyses will be found in Figs. 8 and 9.

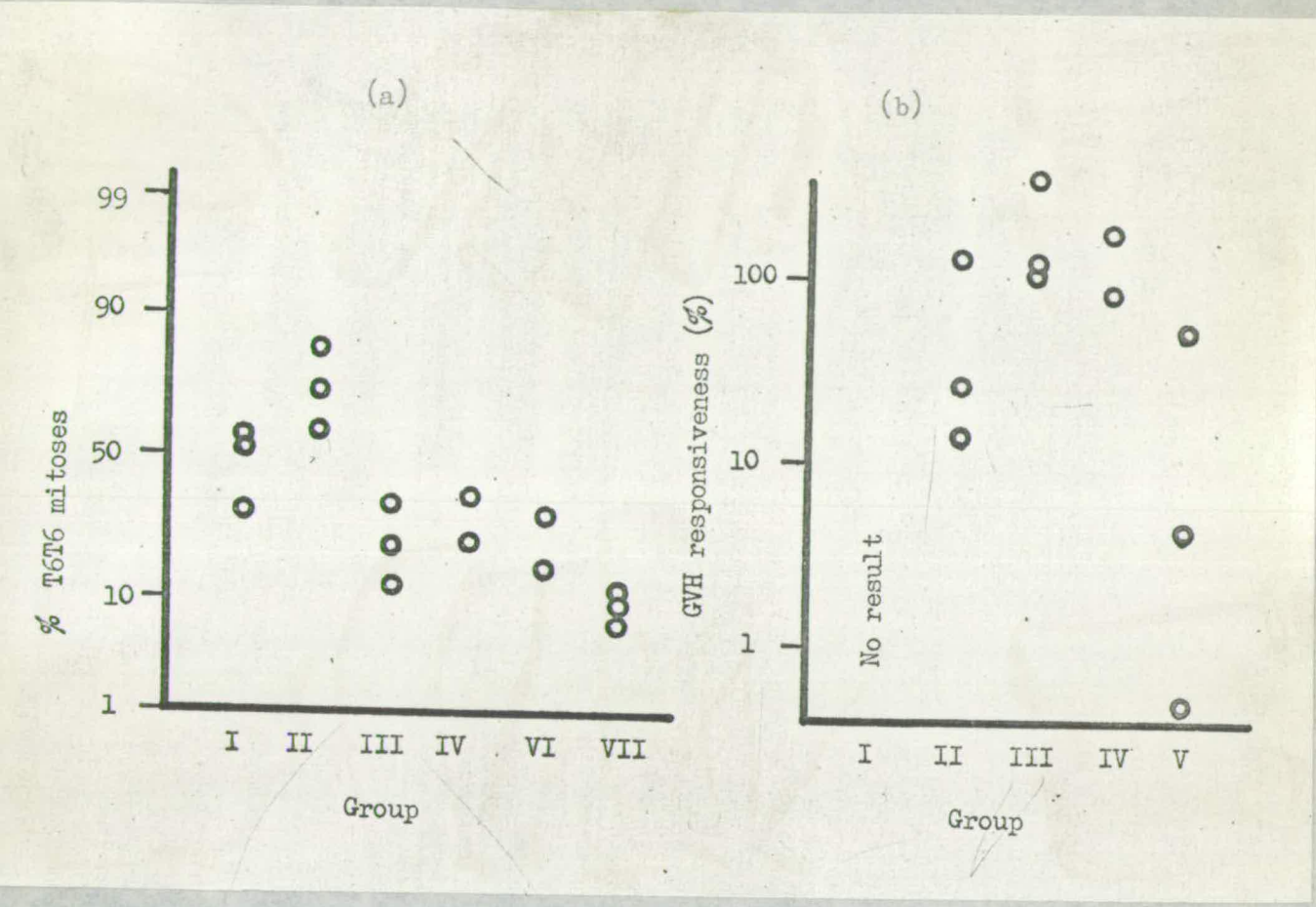


Fig. 8. Restoration of responsiveness. The groups of mice detailed in the legend to Fig. 6 were each split in two. Half were subjected to GVH spleen weight assays in order to estimate overall responsiveness (b), while the other half were used in GVH mitotic tests in order to estimate the percentage contributions of donor (T6T6) and host competent cells to the overall responsiveness (a).

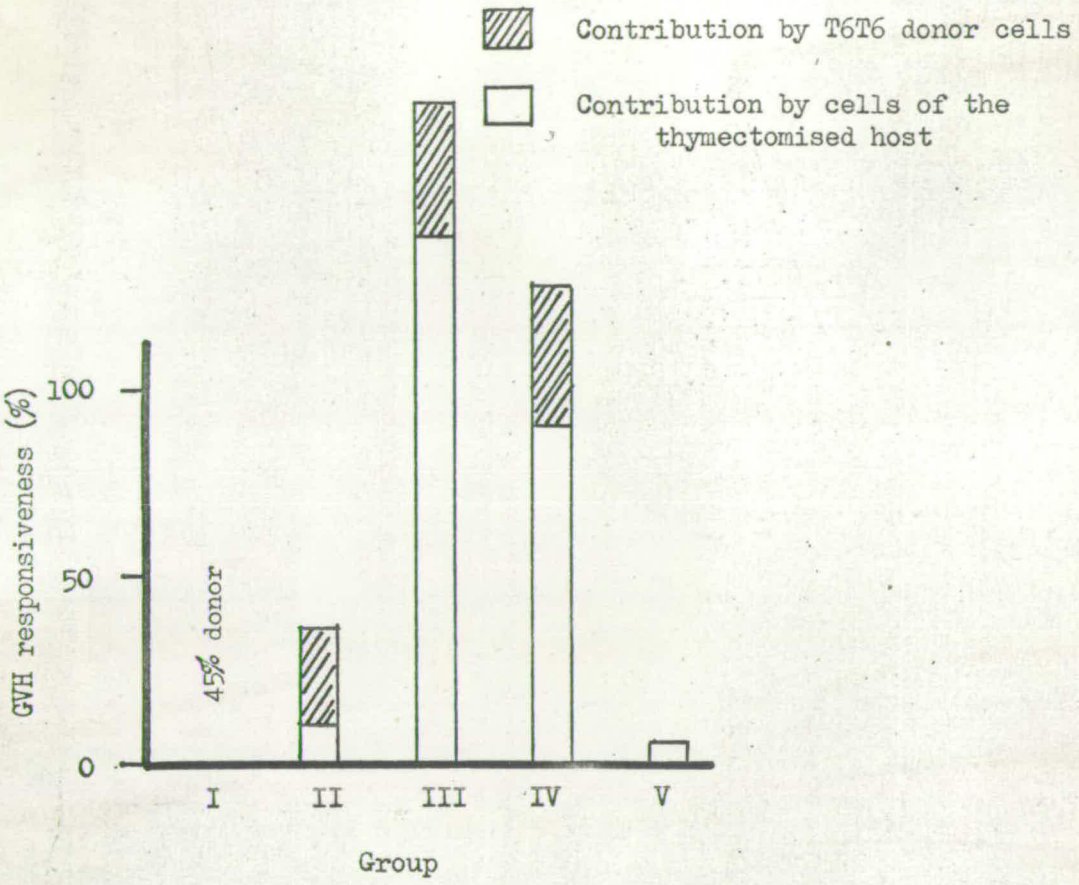


Fig. 9. Restoration of responsiveness. In this diagram the mean proportions of donor and host mitoses from each group in Fig. 8(a) have been superimposed on the mean GVH responsiveness of the same group in Fig. 8(b) to give the result shown in each column above.

mitotic test as described in Part I. They were killed and individual suspensions made of their lymph node cells. These were injected into an equal number of irradiated (C57BL x CBA)$F_1$ hybrid recipients, and chromosome preparations made on the recipient spleens. The three mice which had received unmarked CBA inocula were subjected to GVH spleen weight assay, as described in Part I. These mitotic and spleen weight assays were carried out between 16 and 21 weeks after the transfers of lymphoid tissue had been made. The proportions of T6T6 and $T6^-$ mitoses scored in the karyotype analyses are shown in Fig.8a, and the results of the spleen weight assays in Fig.8b. The mean proportion of T6T6 mitoses for each group has been combined with the mean GVH responsiveness and plotted on a linear scale in Fig. 9.

The spleen weight assays indicate that all the inocula tested had restored some degree of GVH responsiveness to the thymectomised recipients. (Unfortunately the assays for Group I had to be discarded). There was a suggestion that the responsiveness of the mice receiving thymus grafts (III and IV) was higher than that of those receiving dissociated cells (I and II), and this was borne out in the chromosome preparations by observations of mitotic density. Although no measurements were made, this was obviously higher in groups III and IV. In the karyotype analyses both lymph node and thymus cell suspensions had evidently provided substantial proportions of mitoses - rather more in fact than had been provided by the thymus grafts, although these brought about the greater overall recovery of

responsiveness. If these results are taken to reflect the proportions of competent cells bearing the two karyotypes, then the results indicate that the thymus grafts produced a number of competent cells equivalent to 50 million lymph node cells, and that in addition to this they brought about a large increase in the number of competent host cells (Fig.9). The lymph node cells (and probably thymus cells) on the other hand, although providing donor competent cells, did not influence the number of competent host cells. An attempt was made to examine the proportions of T6T6 and T6$^-$ cells dividing in the neonatal thymus grafts. In only one of these was it possible to score the mitotic figures. Of the seventeen mitoses scored, however, all were of host karyotype. The substance of ex eriments 12 and 13a has already been published (Taylor, 1963).

(b) The previous experiment was repeated, but instead of being neonatally thymectomised, the recipients were rendered unresponsive by thymectomy in adult life followed by irradiation, as described in the section on methods. Spleen weight assays were not done; instead an attempt was made to estimate overall immune potency by counting the stathmokinetic indices of the cell suspensions used for karyotype analysis. In the GVH mitotic tests each of the chimaeric cell suspensions was divided between one hybrid and one CBA recipient. If many mitoses were found in the latter it would indicate that a high proportion in the hybrid were due to causes other than the GVH reaction. Forty irradiated-thymectomised CBA/T6T6 male mice were divided into 4 groups and transplanted with various lymphoid tissues from CBA donors as follows:

Table 9. Restoration of responsiveness to irradiated-thymectomised mice by transfer of various lymphoid tissues. The proportions of competent cells of donor and host type have been estimated by the GVH mitotic test. The first donors were CBA, and the irradiated-thymectomised recipients CBA/T6T6 mice.

| Lymphoid tissue transfer | GVH mitotic test: Hybrid recipients. Donor T6T6 | Donor T6⁻ | Donor % | *SI (%) | CBA recipients. Donor T6T6 | Donor T6⁻ | Donor % | *SI (%) |
|---|---|---|---|---|---|---|---|---|
| 1 neonatal thymus graft | 17 | 17 | 50 | 1.28 | 5 | 5 | 50 | 0.08 |
| | 20 | 12 | 38 | 1.06 | 3 | 7 | 70 | 0.05 |
| | 15 | 11 | 42 | 0.82 | Not done | | | |
| 150 million thymus cells, i.p. | 5 | 25 | 83 | 0.74 | 0 | 0 | - | 0.13 |
| | 5 | 19 | 79 | 0.57 | 0 | 0 | - | 0.09 |
| 150 million lymph node cells, i.p. | 1 | 22 | 95 | 0.69 | 0 | 0 | - | 0.26 |
| | 3 | 15 | 83 | 0.83 | Not done | | | |
| No transfer | 0 | 0 | - | 0.00 | 0 | 0 | - | 0.02 |

* SI = Stathmokinetic index.

| Group. | Inoculum. |
|---|---|
| I | 150 million adult thymus cells. |
| II | 150 million adult lymph node cells. |
| III | 1 neonatal thymus graft. |
| IV | No transfer. |

After a further 8 weeks the mice were killed and lymph node cell suspensions were made. A pooled suspension for each group was made from 4 mice, and this was divided among 2 irradiated recipients, one of which was a (C57BL x CBA)$F_1$ hybrid and the other a CBA, so that each received the lymph node equivalent of 2 mice. These mice were killed 3 days later, and their spleens prepared for karyotype analysis and estimation of stathmokinetic index. The results are shown in table 9. As in the previous experiment the thymus grafts brought about an increase in the competence of host cells, while the cell suspensions did not. In addition, it was seen that the dissociated thymus cells, while proliferating rapidly in the hybrid hosts, were no more capable than were lymph node cells of proliferating in syngeneic hosts. Most of the proliferation in the hybrids must therefore result from exposure of the thymus cells to the foreign C57BL antigens.

Origin of lymphoid cells in the embryo: the nature of lymphoid stem cells.

14. Contribution of competent cells by embryonic thymus grafts; effect of irradiating the thymus grafts.

The object of this experiment was to compare embryo thymus grafts with neonatal thymus grafts as sources of competent cells.

after transfer to thymectomised recipients. If differentiation of lymphocytes from the thymus epithelium occurred on a larger scale in the embryo than it appeared to do after birth, then a higher proportion of donor competent cells might be expected in the recipients of embryo than of neonatal thymus. The host mice were irradiated-thymectomised CBA/T6T6, as in the last experiment. Half of them received neonatal thymus grafts, and half 16 day embryo thymus grafts, from CBA mice. Half of the neonatal donors and one pregnant female were irradiated with 900 r immediately before the transfer. Eight weeks later the recipients were killed and their lymph nodes taken for GVH mitotic test. From the results in Table 10, it can be seen that the embryo thymuses had provided some of the mitoses, but in a smaller proportion than was provided by the neonatal grafts. Irradiation reduced the proportion of donor mitoses in both groups, but still permitted the restoration of host cell competence.

15. Repopulation of thymus with embryo liver cells.

The object of this experiment was to try to detect cells of lymphoid potentiality in embryo liver at a stage before lymphoid cells become evident in the thymus. The existence of such cells at this stage would make it possible that even the first-appearing lymphocytes in the thymus might arise by the finishing-school, rather than the germinal mechanism.
The pricipal of the experiment was to mix the embryo cells to be tested in known proportions with adult bone marrow cells (which served as a standard) and to inject the mixture into

irradiated recipients, so as to allow them to proliferate competitively. The proportion of mitoses of "test" origin was then taken as a measure of the proliferative capacity (PC) of the "test" cells relative to normal bone marrow cells. If this was affected differentially in recipient thymus as against bone marrow then that would be evidence that lymphoid and haemopoietic functions were served by different stem cell lines. Recipient mice were either CBA/T6T6 or CBA, irradiated with 900 r immediately before cell transfer. The age of donor embryos was dated from observation of the vaginal plug. The embryo livers were removed and cell suspensions made by gentle pipetting. It is possible that a small quantity of intestinal tissue was included with the liver. The cells were about 70% viable. They were mixed with adult bone marrow cells in various proportions, and injected intravenously. Karyotype analyses were made on recipient thymus and bone marrow at various times after transfer. The results (Table 11, Fig.10) show that the embryo liver cells had a proliferative capacity about equal to adult bone marrow in both recipient thymus and marrow. In one experiment (expt.V, Table 11) the embryo cells were taken from a homogenate of embryo tissue. This was prepared from the trunk region, but taking care to exclude liver and thymus. The cells were dissociated without trypsinisation and only 20% were viable. They had the same proliferative capacity per viable cell as the embryo liver. Although in all the results there was a tendency, with increasing time after transfer, for the proportions of mitoses to drift away from the mean, the proportions in thymus

Table 10. Proportions of competent cells of host- and thymus donor-type in irradiated-thymectomised CBA/T6T6 mice receiving neonatal or embryo thymus grafts. Some of the thymus donors were irradiated with 900 r immediately before the transfer.

| CBA Thymus graft | GVH mitotic test | | |
|---|---|---|---|
| | T6T6 | $T6^-$ | % thymus donor type |
| Neonatal | 11 | 12 | 52 |
| | 19 | 7 | 27 |
| | 18 | 22 | 55 |
| Irradiated neonatal | 11 | 8 | 42 |
| | 12 | 6 | 33 |
| | 27 | 3 | 10 |
| 16 day embryo | 18 | 6 | 25 |
| | 23 | 5 | 18 |
| | 25 | 4 | 14 |
| Irradiated 16 day embryo | 25 | 1 | 4 |
| | 18 | 6 | 25 |
| | 25 | 0 | <4 |

Table 11. Comparisone of proliferative capacities of embryo cells with adult bone marrow cells in the thymus and marrow of irradiated host mice.

| Expt. | Age of embryos | Nos. of cells transferred: CBA embryo liver | CBA/T6T6 adult marrow | Time after transfer (weeks) | Host | Mitoses scored in: Thymus T6T6/T6⁻ | PC (%) | Marrow T6T6/T6⁻ | PC (%) | **P |
|---|---|---|---|---|---|---|---|---|---|---|
| I | 15 days | $10^5$ | $10^6$ | 3 | CBA | 2/15 | 70 | 4/28 | 70 | - |
| | | | | | CBA | 4/29 | 73 | 8/29 | 36 | >0.2 |
| I | 15 days | $10^6$ | $10^6$ | 3 | CBA/T6T6 | 6/9 | 150 | 16/24 | 150 | - |
| | | | | | CBA/T6T6 | 5/8 | 160 | 15/17 | 113 | - |
| | | | | | CBA | 4/3 | 75 | 19/16 | 84 | - |
| I | 12 days | $10^6$ | $10^6$ | 5 | CBA/T6T6 | 12/2 | 17 | 13/2 | 15 | - |
| | | | | | CBA/T6T6 | 14/13 | 93 | *(4) 18/34 | 189 | >0.1 |
| | | | | | CBA *(1) | 25/2 | 8 | *(5) 12/15 | 125 | <0.001 |
| | | | | | CBA *(2) | 8/7 | 88 | 7/8 | 114 | - |
| | | | | 7 | CBA/T6T6 | 5/10 | 200 | 5/10 | 200 | - |
| | | | | | CBA/T6T6 | 4/6 | 150 | 8/7 | 88 | - |
| | | | | | CBA | 3/12 | 400 | 4/11 | 280 | - |
| | | | | 8 | CBA/T6T6 | 14/1 | 7 | 14/1 | 7 | - |
| | | | | | CBA | 9/3 | 33 | 11/4 | 36 | - |
| | | CBA embryo homog. | CBA/T6T6 adult marrow | | | | | | | |
| V | 11 days | 5 X $10^5$ | 5 X $10^5$ | 6 | CBA/T6T6 *(3) | 24/20 | 83 | *(6) 13/28 | 215 | <0.05 |
| | | | | | CBA/T6T6 | 6/14 | 233 | 3/7 | 234 | - |
| V | - | - | $10^6$ | 6 | CBA | 24/1 | 4 | 25/0 | <4 | - |
| | | | | | CBA | 15/2 | 13 | 20/1 | 5 | - |
| | | | | | CBA | 24/1 | 4 | 20/1 | 5 | - |

*Numbers referring to Table 12.

**Probability of difference between thymus and bone marrow ($\chi^2$ test).

PC = Proliferative capacity of embryo cells (Expts. I - IV) or host cells (Expt. V) relative to adult marrow cells.

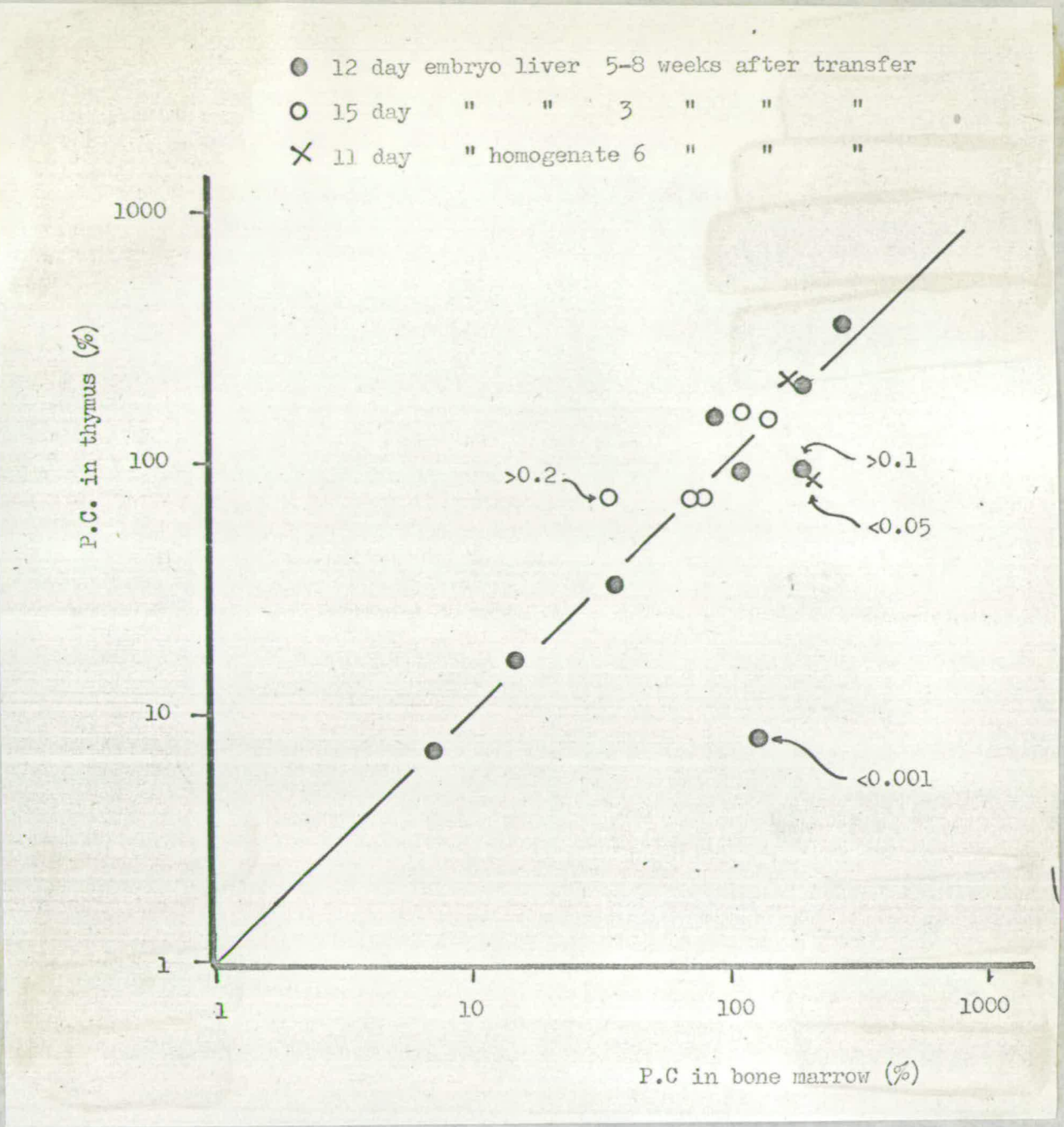


**Fig. 10.** In this diagram the results from Table 11 are presented in graphical form. All values are of proliferative capacity (PC) of embryo cells relative to adult marrow cells. Each point represents one recipient mouse in which this quantity has been estimated in thymus (ordinate) and bone marrow (abscissa). The figure shows a strong correlation between the PC in thymus and bone marrow, although two points deviate significantly.

Table 12. Classification of nuclei in chromosome preparations from Tables 11 and 13.

| No. of preparation ( * in Tables 11 and 13) | | | Round nuclei | Polymorph. nuclei | Mitoses | Polymorphs. (%) |
|---|---|---|---|---|---|---|
| Table 11 | (1)* | Thymus | 1040 | 6 | 6 | 0.57 |
| | (2) | Thymus | 1016 | 8 | 12 | 0.77 |
| | (3) | Thymus | 329 | 7 | 6 | 2.00 |
| | (4) | Marrow | 246 | 50 | 2 | 17.0 |
| | (5) | Marrow | 154 | 44 | 2 | 22.0 |
| | (6) | Marrow | 281 | 131 | 9 | 31.0 |
| Table 13 | (7) | Thymus | 403 | 24 | 8 | 5.5 |
| | (8) | Marrow | 142 | 76 | 4 | 34.0 |

remained in most cases highly correlated with those in bone marrow. In two recipients, however, there was a significant difference between the two tissues in this respect (Fig.10). In view of reports of small foci of granulopoiesis in the thymus of animals recovering from irradiation (Urso and Congdon, 1957; Harris, 1958) the chromosome preparations were examined for granulocytes. The ring or horseshoe forms of granulocyte nuclei could easily be distinguished from round nuclei. While these made up about 25% of the bone marrow cells, they were extremely scarce in most of the thymus preparations, and certainly too few to account for the mitoses of embryo origin (Table 12).

16. Proliferative capacity of bone marrow after irradiation: existence of pluripotential stem cells.

The proliferative capacity of bone marrow can be lastingly impaired by irradiation (Cudkowitz, Shearer, Upton and Hughes, 1964). In this experiment the proliferative capacity of such "depleted" bone marrow was compared with that of normal bone marrow by the method just described. If any dissociation in the capacity to repopulate bone marrow and thymus occurred as a result of irradiation it should be readily detected in this way. The depleted donors were CBA/T6T6 mice which had survived 900 r, without bone marrow, 12 weeks before the cell transfer. The results (Table 13) show that the proliferative capacity of depleted bone marrow cells had been depressed to the same extent (about 0.7%) whether it was assessed in recipient thymus or marrow. Further examination revealed that a proportion of cells had a distinctive chromosome anomaly, additional to the T6

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Table 13. Comparison of proliferative capacities of stem cell-depleted and normal bone marrow cells in the thymus and marrow of irradiated hosts. Karyotype analyses were performed 3 weeks after cell transfer.

| Nos. of marrow cells transferred: | | | Mitoses scored in: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Thymus | | | | Marrow | | | | |
| CBA/T6T6 (depleted) | CBA (normal) | Host | T6T6 | Clone | $T6^-$ | **PC (%) | T6T6 | Clone | $T6^-$ | **PC (%) | ***P |
| $10^6$ | $10^5$ | CBA | 1 | 0 | 35 | 0.3 | 2 | 1 | 33 | 0.9 | - |
| $10^7$ | $10^5$ | CBA | 5*(7) | 33 | 53 | 0.7 | 27*(8) | 7 | 50 | 0.7 | < 0.001 |

*Nos. of chromosome preparations referring to Table 12.

**Proliferative capacity of "depleted" relative to normal bone marrow.

***Probability of difference ~~between~~ in karyotype distribution between thymus and bone marrow ($\chi^2$ test).

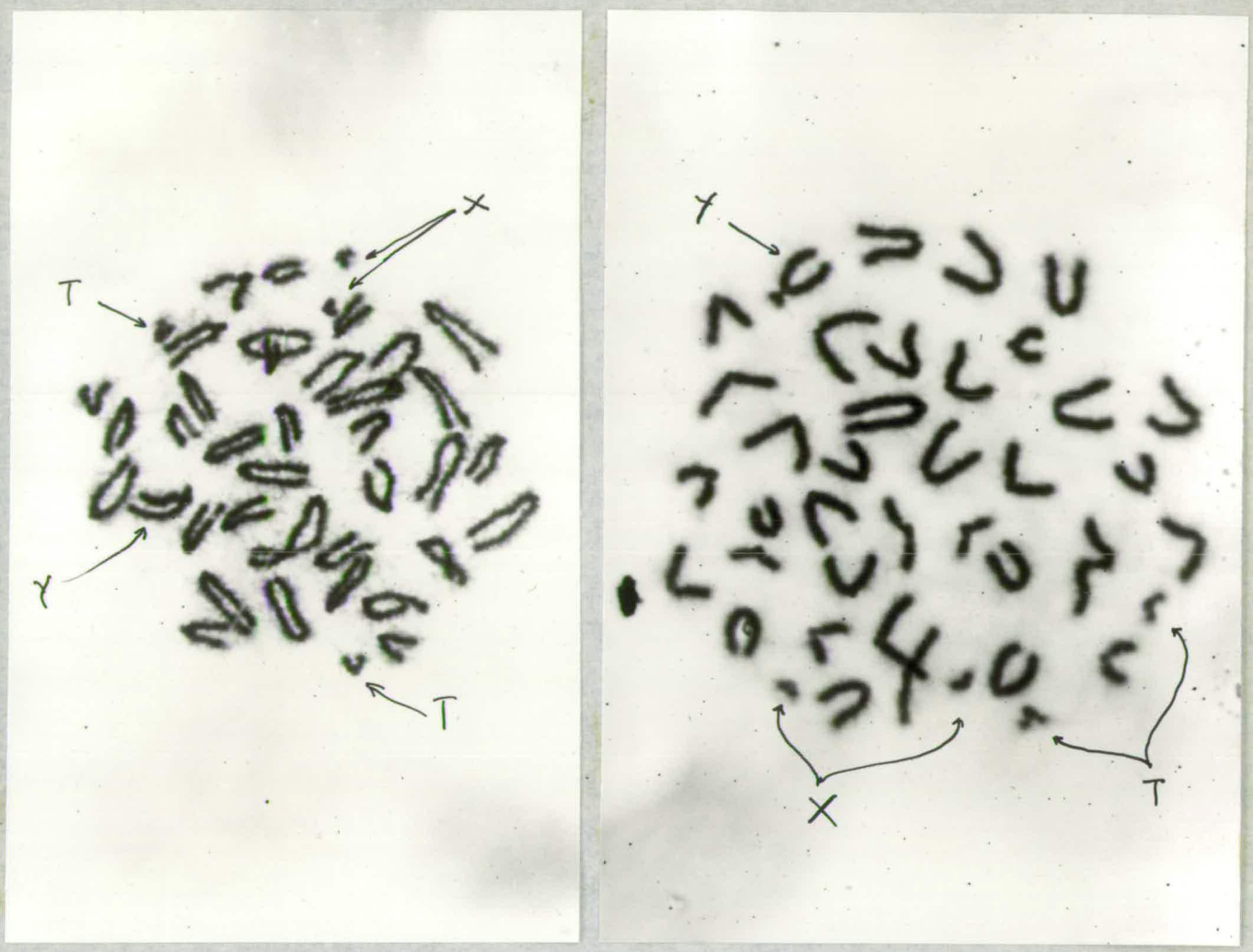


Fig. 11. Chromosomes of two cells belonging to the clone described in the text. A pair of very small chromosomes (X) can be seen in addition to the T6 chromosomes (T). In many cells an anomalous telocentric was also evident (Y).

translocation (Fig.11). These cells were present in both thymus and marrow of the recipients, but were significantly more frequent in thymus.

Immunological responses of thymus cells.

It was shown in experiments 12 and 13 that dissociated thymus cells could restore immunological responsiveness and could themselves become competent. In the first 4 experiments of this section, the potency of thymus cells to perform various immune responses is compared with that of lymph node and spleen cells. For this purpose the use of assay systems similar to the GVH spleen weight assay was considered, but the responses of thymus cells were so low that it would have been impractical to inject enough thymus cells to obtain significantly positive responses at both high and low dose levels. Accordingly, only one dose level of thymus cells was injected, and this was compared against a dose-response line for lymph node and spleen cells on the assumption that the slope of the dose-response line for thymus cells was the same. The last two experiments trace the timecourse of development of responsiveness by mice receiving thymus cells, and compare the effects of intravenous and intraperitoneal injection of the cells.

17. Comparison of potency between thymus and spleen cells in GVH splenomegaly reaction.

Five litters of (C57BL x CBA)$F_1$ hybrid mice containing at least 6 mice in each litter were used. Two in each litter were

injected with 10 million adult CBA thymus cells intraperitoneally, two with 10 million adult CBA spleen cells, and two with 10 million hybrid spleen cells. The mice were killed 7 days later and the spleens weighed. The results are shown in Table 14 In order to estimate relative potency the slope of the dose response line was taken from the mean slope obtained in 20 other assays. In two of the litters the mean spleen index for mice receiving thymus cells failed to reach the level of significance and therefore the true value of relative potency is probably less than the value indicated.

18. Comparison of potency between thymus cells and lymph node cells in GVH mitotic reaction.

A suspension of thymus cells was prepared from CBA mice, and one of lymph node cells from CBA/T6T6 mice. The suspensions were mixed in different proportions and injected intravenously into irradiated hybrid recipients so as to allow the cells to proliferate competitively. ~~Three~~ Two or four days later the recipients were killed and chromosome preparations made from their spleens. The potency of the thymus cells in the production of mitoses was only about 10% of the potency of the lymph node cells (Table 15).

19. Comparison of potency of thymus cells with a mixture of lymph node and spleen cells in the restoration of responsiveness to irradiated mice.

In each of the following experiments a relationship was established between the dose of lymph node and spleen cells transferred and the antibody response obtained. Ideally, in

Table 14. GVH potency of thymus cells relative to spleen cells.

| Hybrid litter | *Spleen indices of test mice receiving : 10 million CBA thymus cells | 10 million CBA spleen cells | Mean log Spleen index: Thymus cells | Spleen cells | Estimate of relative potency, thymus/spleen |
|---|---|---|---|---|---|
| A | 1.44<br>0.93 | 1.76<br>died | 0.063 | 0.246 | <7.2% |
| B | 1.46<br>1.23 | 2.23<br>1.86 | 0.127 | 0.309 | 7.3% |
| C | 1.65 | 1.94 | 0.217 | 0.288 | 36.0% |
| D | 1.03<br>1.07 | 1.89<br>2.08 | 0.022 | 0.297 | <1.9% |

*Spleen index = Relative spleen weight for test mouse / mean relative spleen weight for 2 control mice receiving hybrid cells.

Calculated on the assumption that the slope : increase in log spleen index / increase in log cell dose = 0.16.

Table 15. GVH potency of adult thymus cells relative to lymph node cells compared by mitotic competition after transfer to irradiated recipients.

| Expt. | Cells injected (millions): CBA thymus | CBA/T6T6 lymph node | Days after cell transfer | Hybrid recipients: Mitoses scored T6T6 | T6- | *RP thymus/ lymph node | CBA recipients: mitoses scored T6T6 | T6- |
|---|---|---|---|---|---|---|---|---|
| 1 | 63 | 7 | 2 | 12 | 12 | 10 | 1 | 0 |
| | | | | 19 | 14 | 11 | 1 | 3 |
| | 63 | 7 | 4 | 17 | 13 | 9 | 4 | 2 |
| | | | | 17 | 13 | 9 | 5 | 1 |
| 2 | 100 | 10 | 4 | 20 | 20 | 10 | | |
| | | | | 20 | 12 | 6 | | |
| | | | | | | | not counted | |
| | 50 | 50 | 4 | 25 | 3 | 12 | | |
| | | | | 26 | 4 | 15 | | |

* RP = Relative potency = $\frac{\text{No. T6- mitoses scored}}{\text{No. T6T6 mitoses scored}} \times \frac{\text{No. lymph node cells injected}}{\text{No. thymus cells injected}}$

X 100

the construction of these dose-response curves for the primary response the numbers of cells transferred at each dose level should be made up to a constant number by admixture of cells made unresponsive in some way - e.g. by paralysis. However, the dose response curves obtained without such admixture were approximately straight and thus the error made in this way was likely to be small.

(a) Primary response to STH.

Forty-eight CBA mice were irradiated with 900 r and eight left without irradiation. The irradiated mice were divided into 6 groups, and on the following day injected with various numbers of cells intraperitoneally. Five groups received mixed CBA lymph node and spleen cells in doses which increased by five-fold steps from 0.8 millions to 100 millions. One group received 100 million CBA thymus cells, and one received no transfer. All the mice were then challenged with STH, and bled 7 days later. The agglutinin titres obtained can be seen in Fig. 12. Although most of the responses of the recipients receiving thymus cells were not distinguishable from those of the controls receiving no cell transfer, two responses were fairly high.

(b) Primary response to BSA.

In order to obtain a satisfactory response to this antigen it is necessary to allow a long time to elapse after immunisation. During this time the immune mechanisms of the irradiated hosts would have an opportunity to recover. In order to prevent this recovery half of the recipients were thymectomised before irradiation. A dose-response curve was first

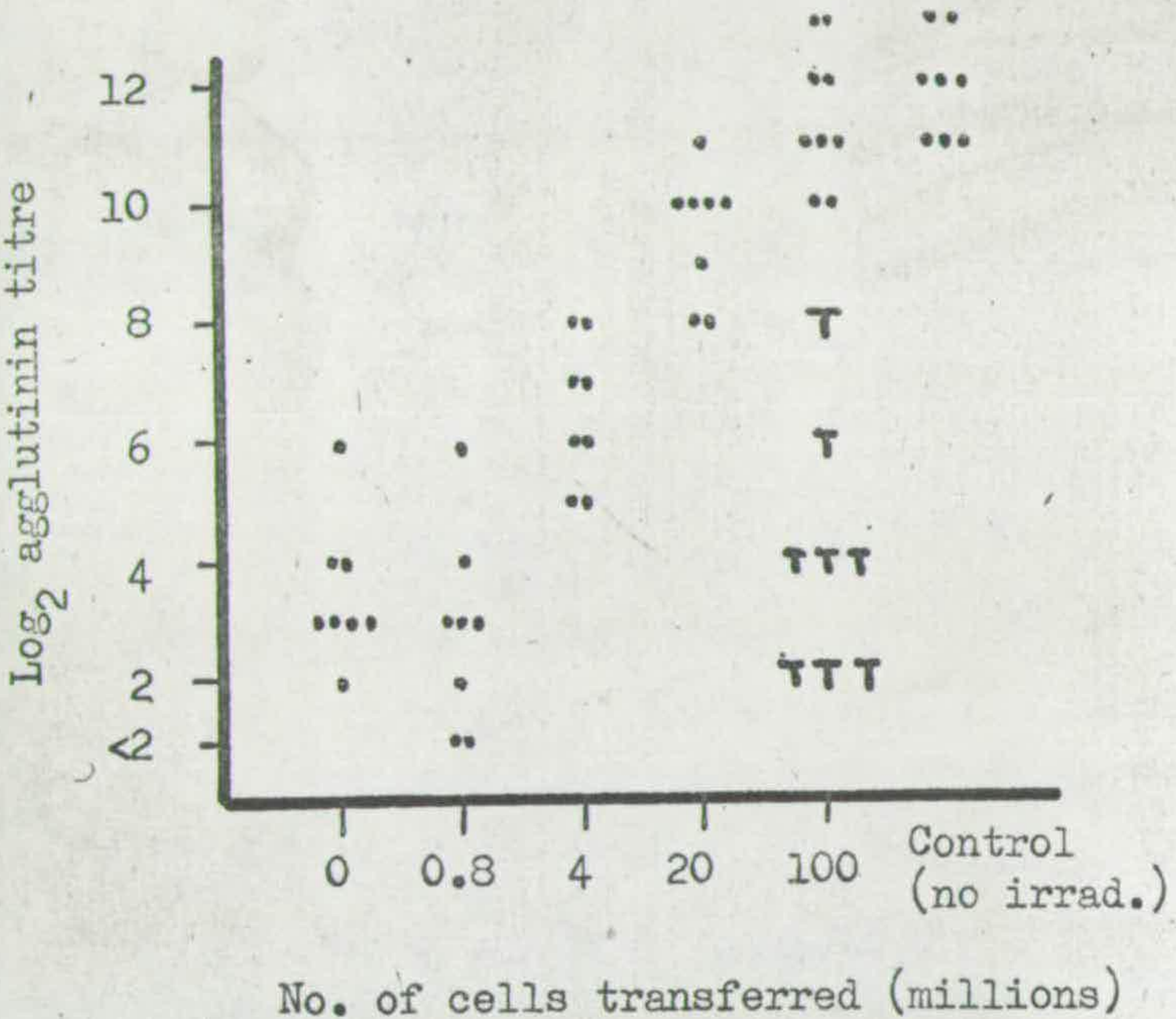
Log2 agglutinin titre
12
10
8
6
4
2
<2
0
0.8
4
20
100
Control
(no irrad.)
No. of cells transferred (millions)

Fig. 12. Primary response assay with STH. Recipient mice were irradiated with 900 r and injected with mixed spleen and lymph node cells (dots), or with adult thymus cells (T), and then challenged with STH. The diagram shows that as few as 100 million spleen and lymph node cells are sufficient to produce responses equivalent to those of unirradiated mice. The thymus cells were less than 4% as potent in this system.

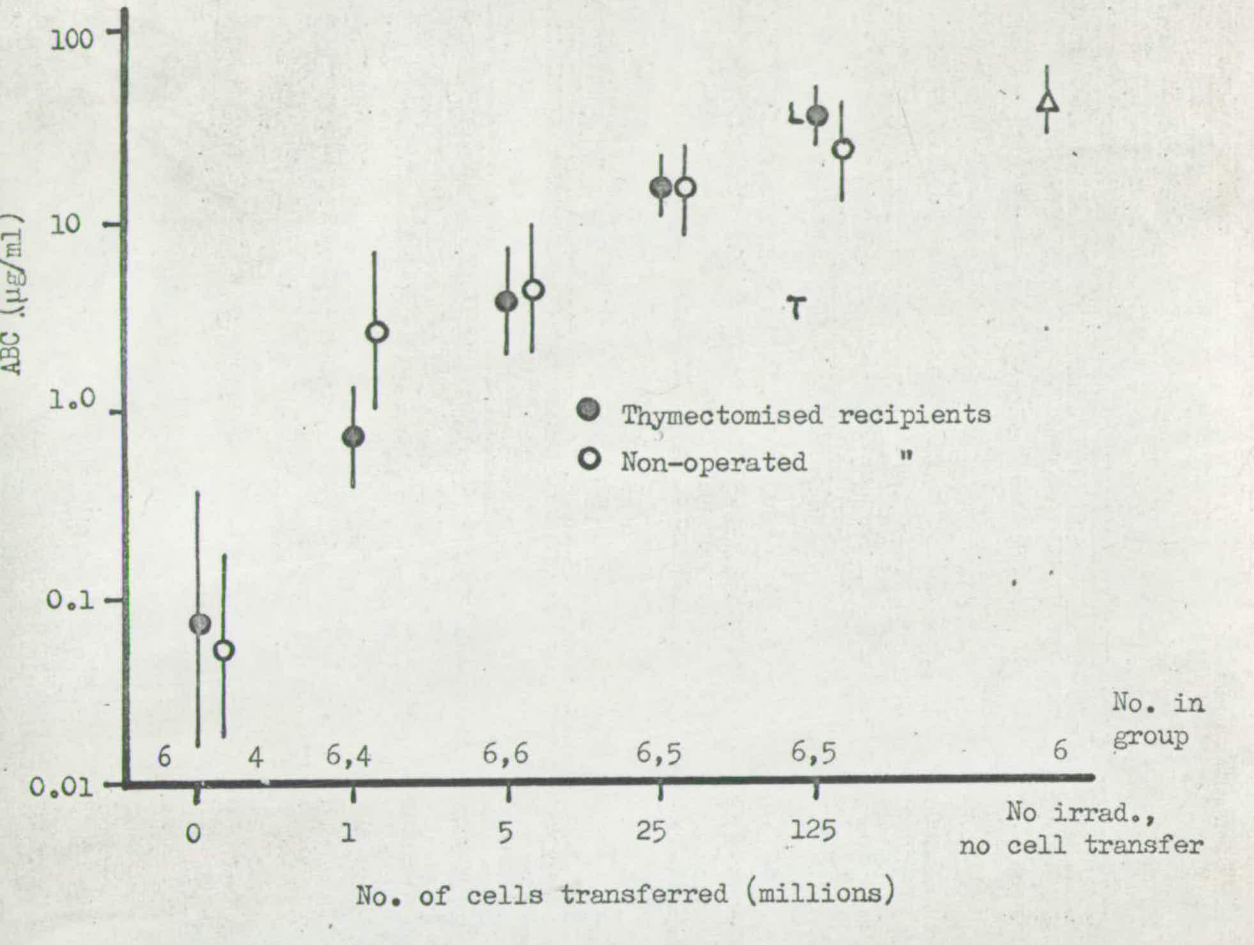
100
10
1.0
0.1
0.01
ABC (µg/ml)
Thymectomised recipients
Non-operated "
No. in group
6
4
6,4
6,6
6,5
6,5
6
0
1
5
25
125
No irrad., no cell transfer
No. of cells transferred (millions)

Fig. 13. Primary response assay with BSA. Recipient mice (either thymectomised or non-operated) were irradiated and then immediately injected with mixed spleen and lymph node cells, and challenged with BSA Freund's. The diagram shows the relation between the cell dose and the antibody titres at 28 days after challenge. The mean responses of mice receiving lymph node cells (L) and thymus cells (T) from another similar experiment (see Fig. 16) have been plotted here for comparison. The thymus cells were about 4% as potent as lymph node cells.

established as follows: thirty adult CBA mice were thymectomised and 36 left unoperated. Four weeks later they were divided into groups of six and all groups except one irradiated with 900 r and injected with bone marrow to ensure survival. The irradiated mice were injected on the same day with different doses of CBA lymph node and spleen cells as detailed in Fig.13, and challenged with BSA Freunds on the following day. The antibody titres at 28 days after challenge can be seen in Fig. 13. The degree of recovery in the unoperated animals was slight at this time, but became more obvious at 42 days after challenge (no Fig. given). The responses of thymus cells in a similar situation form part of another experiment (experiment 21b), and the relevant results have been entered on Fig.13 for comparison with the dose-response curve. The thymus cells produced responses equivalent to 4 million lymph node cells - i.e. they were about 4% as active on the average, but as will be noted later, the responses were highly variable.

20. Secondary response to BSA.

The object of this experiment was to investigate the capacity of thymus cells to transfer secondary responsiveness, and to use the result as a guide to the degree of primary immunisation which the cells had undergone <u>in situ</u>. As before, a dose-response curve was constructed. The donors were mice immunised with BSA Freunds 3 months previously. Lymph node cell suspensions were made from these, and also from non-immunised donors. Different doses of immunised cells were all made up to 128 millions with non-immune cells, and injected into

normal recipient mice. These were injected with 0.1mg. BSA in saline 5 days later and bled 10 and 20 days after this injection. The titres of antibody found at the 10 day bleed are shown in Fig.14. The responses of thymus cells were examined in an analogous experiment, in which ~~either~~ thymus ~~or~~ lymph node or spleen cells from immunised donors were transferred. The results are shown in Table 16. Since the basal response of the hosts formed a substantial fraction of the total response, it was subtracted from the responses of mice receiving lymph node and thymus cells before these were entered in Fig. 14 for comparison with the dose response curve. The thymus cells were about 3% as active as lymph node cells in this system.

21. Development of responsiveness by unresponsive mice receiving injections of thymus cells.

In these experiments, thymus or lymph node cells were transferred to recipients made unresponsive either by neonatal thymectomy or by thymectomy in adult life followed by irradiation, and the recipients were challenged at various time intervals in order to trace the time course of the development of responsiveness.

(a) Neonatally thymectomised recipients.

Sixteen mice, which had been thymectomised at 3-5 days, were divided at the age of 6 months into four groups. The mice in three of these groups were each injected with 140 million adult thymus cells intraperitoneally, while the remainder received no cell transfer. A group of 6 non-thymectomised littermates was also included. One group receiving thymus cells was

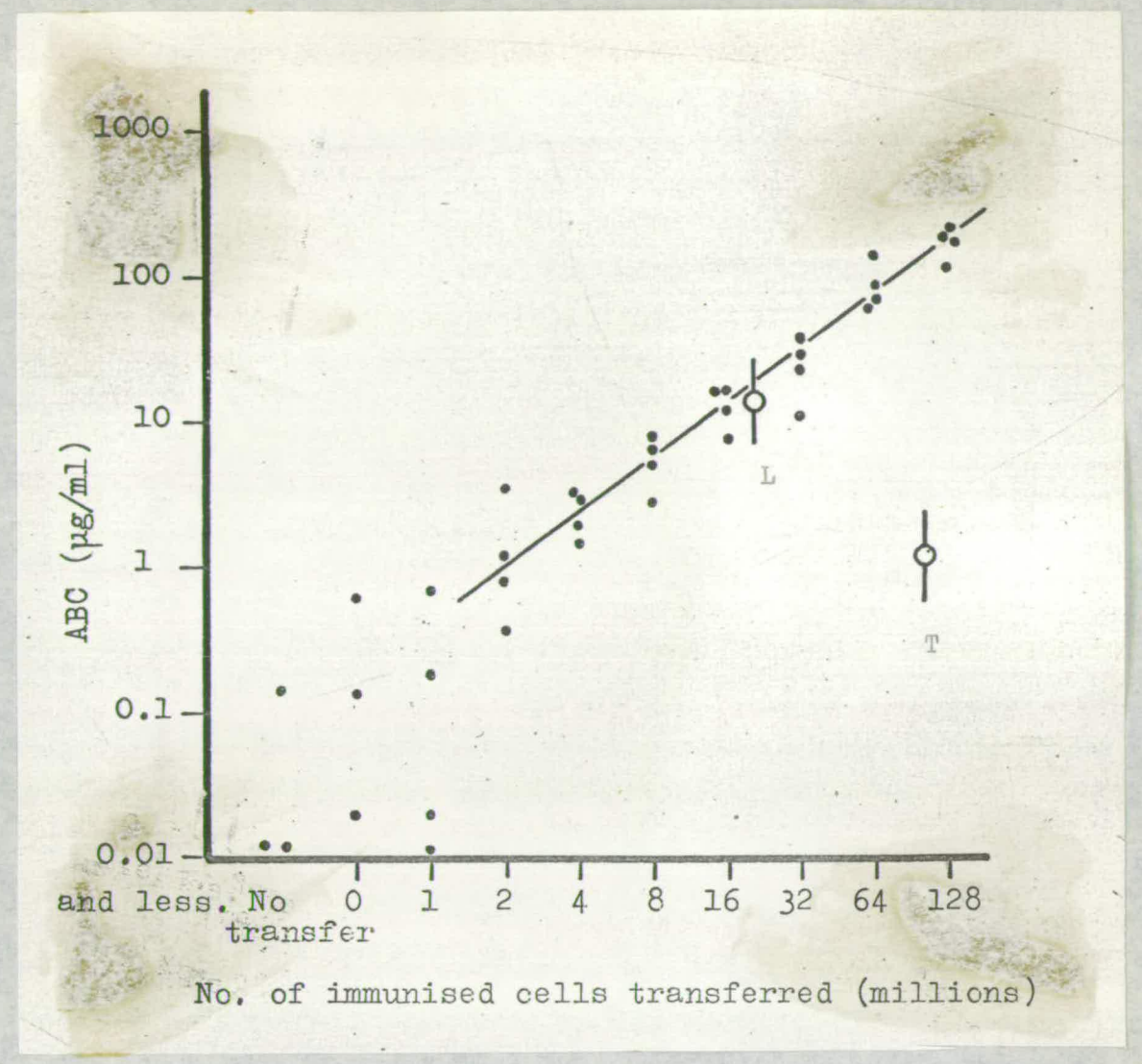


Fig. 14. Secondary response assay with BSA. This diagram shows the relation between antibody produced in adoptive secondary response and the dose of immunised rat lymph node cells. The total number of cells was made up to 128 million by adding non-immunised cells in each group except those receiving "no transfer". Dots are responses of individual mice. Open circles show results from another similar experiment (see Table 16). The mean responses of mice receiving lymph node cells (L) and thymus cells (T) are plotted here for comparison. The thymus cells were about 3% as potent as lymph node cells.

Table 16. Adoptive secondary response to BSA by thymus, lymph node and spleen cells.

| Cells injected | No. in group | Mean antigen binding capacity (μg/ml) at: 10 days | P | 20 days | P |
|---|---|---|---|---|---|
| 100 million thymus | 7 | 2.43 | < 0.02 | 2.56 | < 0.001 |
| 20 million lymph node | 7 | 11.8 | | 11.0 | |
| 20 million spleen | 6 | 5.6 | | 8.87 | |
| None (host primary response) | 7 | 1.06 | | 1.26 | |

Probability of difference between responses of mice receiving thymus cells and the untransferred hosts.

challenged with BSA Freunds at each of the intervals 0, ~~4~~ 1 and 6 weeks after cell transfer, while the 2 control groups were challenged at 6 weeks. They were bled 28 days after challenge. From the results, shown in Fig. 15, it can be seen that the recipients of thymus cells produced little response at either 0 or 1 week, but at 6 weeks the responses approached those of non-thymectomised mice.

(b) Irradiated-thymectomised recipients; differential effect of intraperitoneal and intravenous transfer of thymus cells.

Eighty-eight mice were thymectomised at the age of 10 weeks. Two months later they were irradiated with 900 r, injected with syngeneic bone marrow, and divided into eleven groups. One group of normal mice was included as a control. Most of the mice were injected intraperitoneally with 100 million thymus or lymph node cells, but in two groups, thymus cells were injected intravenously. The first cell transfer took place 2 months after the recipients had been irradiated, and subsequent transfers were arranged so that all the mice could be challenged on the same day, while the interval between cell transfer and challenge varied from 0 to 7 weeks. They were bled 28 days after challenge, which was with BSA-AP. The details of the experiment and the results can be seen in Fig.16. As before, the recipients of thymus cells given intraperitoneally made little antibody when challenged immediately, but their capacity to respond increased subsequently to reach a plateau by three weeks after the cell transfer. After intravenous injection, on the other hand, the responsiveness was

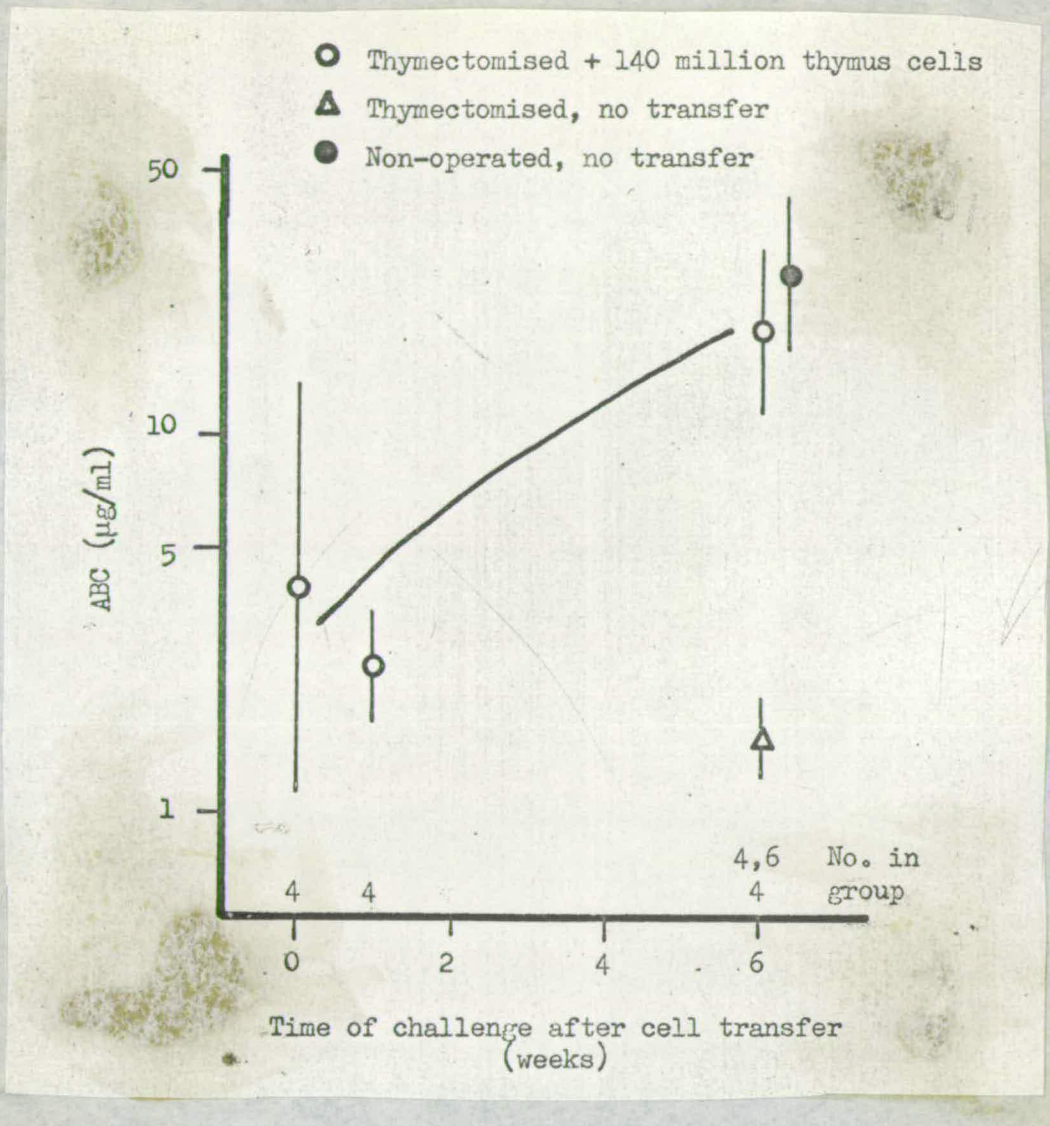


**Fig. 15. Development of responsiveness to BSA in mice neonatally-thymectomised and injected at the age of 6 months with 140 million thymus cells.**

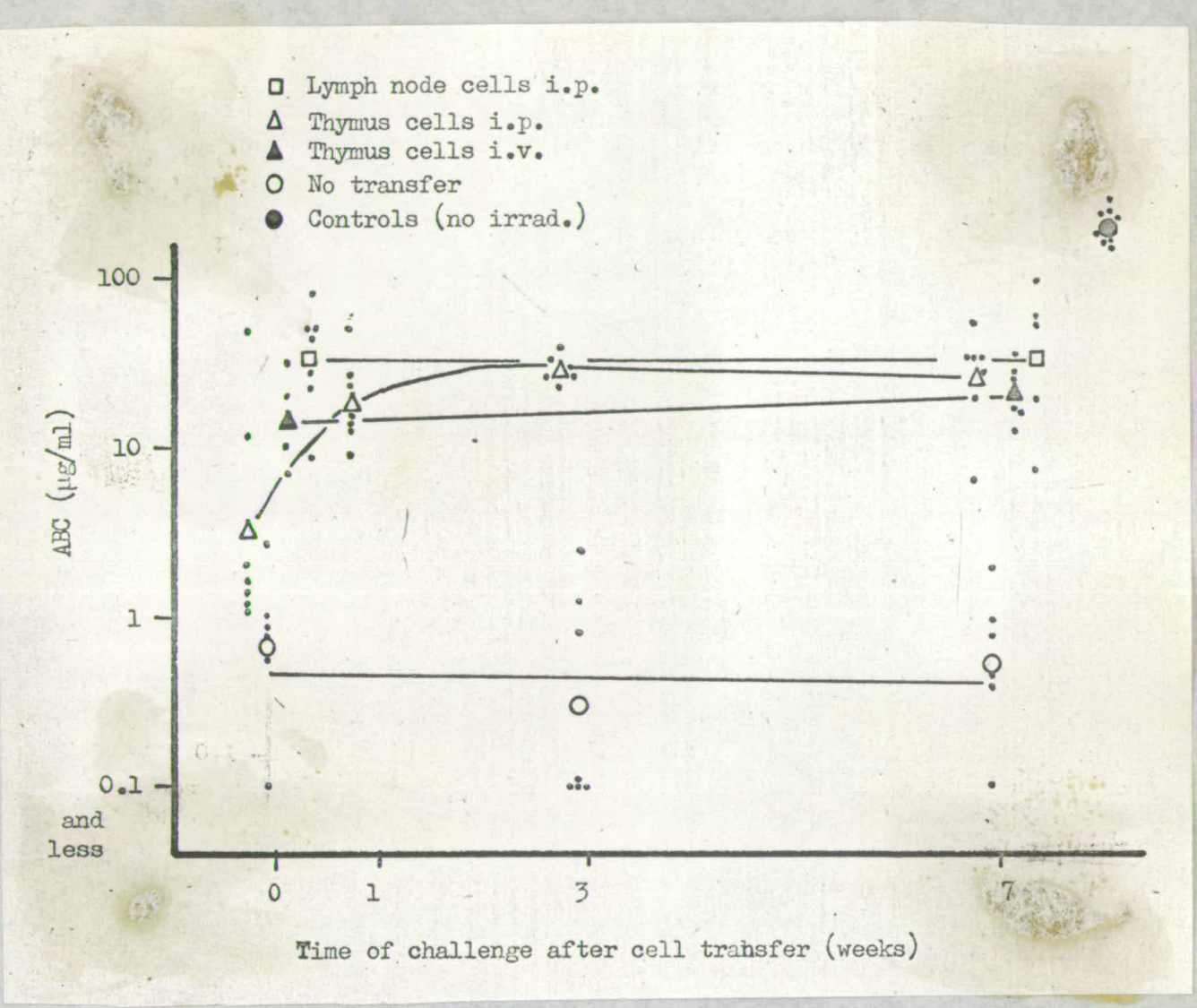


**Fig. 16. Development of responsiveness to BSA in irradiated-thymectomised mice with time after transfer of thymus or lymph node cells. An increase in response can be seen during the first week after intraperitoneal transfer of thymus cells. After intravenous transfer the response was maximal at the first challenge - as it was with lymph node cells.**

maximal at the first challenge. The responses of the mice receiving lymph node cells did not change, as between 0 and 7 weeks after transfer. The individual responses are plotted in order to indicate the pattern of variability. In the group which was challenged immediately after intraperitoneal transfer of thymus cells, most of the mice made little antibody, but the responses of two reach a level equal to those obtained after intravenous transfer. A similar pattern was seen in the previous experiment, (Fig.15), and in experiment 19a where the challenge was with STH (Fig.12). The GVH splenomegaly responses noted after intraperitoneal transfer of thymus cells were also extremely variable (experiment 17), while those in the mitotic competition experiment (no.18) in which the injection of cells was made intravenously were less so.

Pathology of neonatally-thymectomised mice.

No systematic histological study was made, but the following general points were noted. The condition of mice thymectomised at less than 7 days old was often poor, and growth was retarded. Thymectomy at less than 2 days resulted in the development of this "wasting disease" in the majority of mice. The condition did not develop at once but became noticeable some time after weaning. Thereafter it became steadily worse and often resulted in early death. The appearance of most organs was normal, except the lymph nodes and spleen. The former were usually smaller than normal, but could be larger - especially in mice thymectomised later than the first 7 days. The spleen was little altered in size but often had a homogenous red appearance as if lacking white pulp.

The liver sometimes exhibited lesions. In histological sections the main abnormality of lymph nodes and spleen was a marked reduction in the numbers of small lymphocytes. Mice thymectomised at more than 7 days of age rarely developed any severe wasting condition, but they were eventually distinguishable from the controls by certain pathological features, which will be described in Part III. The thymectomised mice used in experiments 12 and 13 were showing signs of wasting (ruffled fur and hunched posture) at the time they received the transfers of lymphoid tissue. At the time they were killed, it was noted that those receiving either neonatal or adult thymus grafts had recovered their normal condition, but those receiving lymph node or thymus cells had not improved noticeably.

The grafts of neonatal thymus in experiments 12 and 13 weighed 15 - 25 mg at autopsy 16 - 21 weeks after grafting. There was no difference in size of the grafts between thymectomised hosts (group III) and the sham-operated hosts (group VII). The grafts of adult thymus were spread out under the kidney capsule and thus could not be weighed. In histological sections the architecture of the neonatal grafts appeared normal, while the adult grafts were reduced to a thin layer of lymphoid cells in which cortex and medulla could not be distinguished.

# DISCUSSION.

## Effect of neonatal thymectomy on immune responsiveness.

The first published reports on the effects of neonatal thymectomy appeared in 1961: Archer and Pierce found that thymectomy of young rabbits much impaired their capacity to produce antibody to BSA, while Miller thymectomised mice on the first day of life and found that they would accept skin allografts indefinately. Since then the effect of neonatal thymectomy has been tested on many species of animals and with many different antigens and manifestations of immunity. Almost invariably the result has been a depression of the immune response, and the results just presented are in agreement with this. An exception however was seen in the results of challenge with STH (experiments 7 - 9). Although in one experiment significant depression of the response was obtained after thymectomy as late as 7 - 14 days, in another experiment no depression was found in mice operated within the first 2 days of life. Similarly variable results were seen by Humphrey, Parrott and East (1964). It can only be speculated that early encounter with bacterial antigens related to STH might have built up a high level of secndary responsiveness to this antigen. Certainly, an abundance of plasma cells is often found in thymectomised animals (Parrott and East, 1962; Arnason et al, 1962), but it is not known if these synthesize specific antibody.

## Effect of age at thymectomy.

As might be expected from the ineffectiveness of thymectomy in adult life the effect of neonatal thymectomy falls off with

increase in the age at which the operation is performed. This has been observed for allograft rejection by Miller (1962 a,b). The weaker the histocompatibility difference the later was the effect of thymectomy demonstrable. The same effect was confirmed here for the response to BSA, and a severe depression of responsiveness was observed even when thymectomy was delayed until 7-14 days. In rats, Arnason et al (1962) observed depression of the response to BSA after thymectomy as late as 3 weeks.

Effect of Sex.

The effect of neonatal thymectomy on the response to BSA was slightly greater in male than female mice (experiment 2). A much more dramatic sex difference has been described in hamsters (Sherman and Dameshek, 1963) and was sex-hormone-dependent (Sherman, Adner and Dameshek, 1963).

Quality of the immune response.

Any qualitative differences in the immune responses of thymectomised animals might suggest that thymectomy had arrested the maturation of individual lymphoid cells at an incomplete stage. The absence of qualitative differences on the other hand would be consistent with the hypothesis that the acquisition of competence is an all-or-none phenomenon. Little information is available on this aspect. Arnason et al (1962) found that both delayed hypersensitivity and antibody production to BSA were depressed by thymectomy in rats. Humphrey et al (1964) found that a few of their mice produced a relative, and occasionally an absolute excess of $\gamma_1$A-globulin

but they did not relate this to specific antibody production. The finding in experiment 10 of normal avidity in anti-BSA sera from thymectomised mice is consistent with an all-or-none hypothesis.

Does the defect lie in the lymphoid cells themselves?

The results of cell transfer studies all concur in the demonstration that on the one hand the lymphoid cells of the thymectomised mouse are defective in their immune responses and on the other that the neonatally-thymectomised mouse is perfectly capable of supporting immune reactions by cells transferred from normal mice. Dalmasso, Martinez and Good (1962) showed that spleen cells from thymectomised mice were defective in their capacity to cause the GVH splenomegaly reaction; while Miller (1962a) found that thymectomised host mice would support a GVH reaction against themselves by cells from normal donors. In addition Miller found that presensitized (1962a) or normal (1963) syngeneic lymph node or spleen cells injected into neonatally thymectomised mice bearing a skin allograft would cause rejection of the graft, and prevent the onset of wasting disease. The same conclusions are indicated for antibody production by experiments 11 - 12 just described, and for GVH reaction by experiment 13.

There is by now considerable evidence that, at least in mammals, the cells competent to react specifically to foreign antigens are lymphocytes. That lymphocytes are necessary for both allograft rejection (Gowans, McGregor, Cowen and Ford, 1962) and for antibody productions (Gowans and McGregor, 1963) was

shown by the abolition of these responses after thoracic duct drainage and its restoration by infusion of lymphocytes. That lymphocytes alone may be sufficient to initiate an immune response against tissue antigens was suggested by the finding that these cells will enlarge, divide and initiate tissue damage when placed in contact with allogeneic lymphocytes in vitro (Bach and Hirschhorn, 1964). For antibody production, however, macrophages may also be necessary, but in non-specific capacity, (Fishman 1962). That small lymphocytes play an active part in GVH reaction was shown by the fact that a proportion of them transformed into large pyroninophilic cells and commenced rapid proliferation in the host spleen (Gowans et al, 1962). The deficiency of small lymphocytes in neonatally-thymectomised animals, which is the principal defect seen in histological studies of their lymphoid tissues, (Miller, Marshall and White, 1962), is consistent with the imputation of competence to these cells.

The origin of the competent cells.

(a) Finishing school hypothesis.

Much of the evidence concerning cell migrations has been obtained from the study of the "homing" of cells transferred to irradiated recipients. While the results obtained with this procedure do not necessarily reflect the normal state of affairs, the behaviour of cell suspensions from diverse sources differed in a clear-cut manner. Lymph node cells (Ford and Micklem, 1963) or thoracic duct cells (Gowans, 1959; Gesner and Gowans, 1962) would repopulate lymph nodes and spleen, but would not enter thymus or bone marrow in significant numbers. Thymus cells

behaved in a similar way: they did not enter the thymus (Ford and Micklem, 1963). Bone marrow cells on the other hand repopulated both marrow (Lindsley, Odell and Tausche, 1956) and thymus (Law, 1952; Ford and Micklem, 1963). They eventually repopulated the lymph nodes also, but took longer to do so than did lymph node or thymus cells (Ford and Micklem, 1963). The γ-globulin of such chimaeras was almost entirely of marrow-donor type (Grabar, Courçon, Barnes, Ford and Micklem, 1962). It was thus established that bone marrow cells are capable of transformation into functional lymphoid cells, and the possibility that some of them might pass via the thymus in doing so was strongly suggested.

Meanwhile, the evidence from thymus-grafting experiments did not contradict this idea. Miller (1962a) using the T6 marker, found some 15% of thymus-donor type cells dividing in the spleen of thymectomised recipients, while in the thymus grafts themselves he found that the majority of ~~xxx~~ cells were of host type. The replacement of dividing cells in the thymus graft by cells of host type is not peculiar to thymectomised hosts, since it also occurred where the host mice were not thymectomised (Wakonig-Vaartaja and Metcalf, 1963). In the present work (experiments 13a and b) these findings are confirmed, while the proportions of donor and host type cells scored in lymph nodes have been made more representative of the competent cell population. Although this revealed a higher proportion of donor cells than was found by Miller, the host-type competent cells were still in te majority. In fact, since the overall responsiveness of the mice receiving thymus grafts was higher than that of mice receiving lymph node cells, while the proportion of donor competent cells was lower, there can be little doubt that the thymus grafts had a faculty, not

possessed by lymph node ~~cells~~ or thymus cells, of bringing about an increase in competent cells of host type. The passage of large numbers of cells from the graft to the host lymph nodes, and their replacement in the graft by cells of host-type strongly suggests directed migration rather than random mixing.

Recently, compelling evidence that an afferent stream of cells enters the thymus in situ has been provided in the experiment of Harris, Barnes, Ford and Evans (1964). By parabiosis of syngeneic mice differing only in the presence or absence of the T6 chromosome these authors were able to avoid both irradiation and thymus grafting, with the possible artefacts which these operations might incur. They found that the dividing cells in the thymus of each partner were partly replaced by cells of the other partner's karyotype. Finally, demonstration of the path of migration has been completed in the regrafting experiment of Harris and Ford (1964). They showed that cells which had entered a thymus graft from its first host were able, after retransplantation of the graft, to populate the lymph nodes of the second host, while fresh cells from the second host replaced them in the thymus graft.

There can be little doubt that a stream of cells does pass through the thymus, in accordance with the finishing school hypothesis, but the occurrence of the mechanisms proposed by the other two hypotheses is not quite precluded, although there are reasons to suppose that neither is very important. These will now be discussed.

(b) Germinal hypothesis.

In the regrafting experiment mentioned above the cells of the thymus graft and the first and second hosts were all separately distinguishable by the presence of one, two or no T6 chromosomes. It was thus possible to show that, by sojourn in the first host, the graft had lost very nearly all its own dividing cells and was not able to contribute more than a very few of these to the lymph nodes of the second host, although (as mentioned above) it was capable of passing on the cells which it had acquired in the first host. Therefore it is likely that the thymus, at least in post-natal life, does not hold a permanent reserve of stem cells within itself as postulated by the germinal hypothesis, but is continuously supplied with these from an outside source.

(c) Humoral hypothesis.

There is now evidence that, even when denied cellular exchange by enclosure in a diffusion chamber, the thymus can still restore some degree of responsiveness to thymectomised mice, and prevent the onset of wasting disease (Osoba and Miller, 1963; Levey, Trainin and Law, 1963). The capacity of the thymus to produce a non-cellular factor which can influence lymphoid maturation is thus established. What is still in doubt is whether this factor is released in significant quantities under normal conditions, or whether it acts mainly within the thymus. The latter hypothesis would require that the proportion of host-type cells found in the lymph nodes after thymus-grafting, as in experiment 13, be entirely accounted for by the finishing school mechanism. The following considerations

suggest that this is so.

Firstly there is the finding of Harris and Ford (1964) that donors and host-type cells within the thymus graft are functionally equivalent and that there is a steady "feeding-through" of cells to the lymph nodes. Secondly the rate of feeding-through is likely to be constant (once the thymus graft has become established) since thymic lymphopoiesis is largely autonomous in that it is unaffected by resection of other lymphoid tissues, partial thymectomy or the presence of up to 23 thymus grafts (Metcalf, 1964). Thirdly, the donor cell population in the thymus graft becomes almost completely replaced by host cells within three weeks (Metcalf and Wakonig-Vaartaja, 1964). Thus in experiment 15a the production of donor-type cells must have ceased by three weeks after grafting, leaving about 16 weeks for host-type cell production. Yet the proportion of host-type found in the lymph nodes was barely enough on this basis to fulfil these predictions. Thus if any cells mature outside the thymus they must be a small proportion of the total. The proportion of host cells was even smaller in experiment 13b but this may only reflect the inadequacy of narrow therapy in replacing stem cells destroyed by irradiation.

Origin of lymphoid cells in the embryo.

The origin of lymphocytes in the embryo has long been a point at issue. Of the early histologists some, following Kölliker (1879), have held that they stem from the epithelial cells of the thymus rudiment; while others, of whom Maximow (1909) was among the first exponents, argued that the lymphoid

cells entered the thymus from outside. Other evidence was based on the behaviour of explants of embryo thymus epithelium denied of cellular exchange. The interpretation of this type of experiment suffers from the following objections: Where lymphoid differentiation was not seen (Gregoire,1935) this may have been due to the inadequacy of the nutritional medium; where on the other hand it was obtained (Auerbach, 1961) this is still no proof that the lymphoid cells originated from the thymus epithelium itself and not from a few immigrant lymphoblasts which went undetected at the time the explant was removed from the embryo. In further support of the immigration hypothesis Gregoire (1935) found that the mitotic index among epithelial cells was far too low to account for the initial influx of lymphocytes - indeed even the overall mitotic index was too low.

The results of experiment 15 indicate that cells capable of repopulating the thymus exist in the embryo at stages before lymphopoiesis becomes evident in the thymus. This is not seen in the mouse until 14 days (Ball and Auerbach, 1960). If haematogenous tissue thus contains lymphoid stem cells at this early stage, it seems probable that in the embryo lymphopoiesis follows the finishing school mechanism, as it does in later life - although differentiation of thymic epithelial cells cannot be excluded at present.

In experiment 14 the embryo thymus grafts donated a smaller proportion of competent cells to the host than did neonatal grafts; and yet they still had the power to raise host cell competence.

Thus if any lymphoid cells are derived from the epithelium they are unlikely to contribute any large part to the competent cells of the body. It is not excluded, however, that a lymphoid type of cell, derived from the epithelium, may play some other role not directly concerned with immune reactions. Irradiation further depressed the donor cell contribution but did not inhibit it completely. This agrees with Auerbach's (1963b) finding that lymphopoiesis in thymic epithelial explants would still proceed after 900 r given in vitro.

The nature of lymphoid stem cells.

The evidence that lymphoid cells arise from stem cells in the bone marrow has already been discussed. The question arises whether these cells are the same as the haemopoietic stem cells (Yoffey, 1960) or whether they form a distinct population. Dissociation of haemopoiesis from lymphopoiesis has been reported by Berman and Kaplan (1959) who found that treatment of bone marrow in vitro with urethane or irradiation impaired its ability to repopulate thymus more than its ability to repopulate marrow. However, the same result was obtained by Urso and Congdon (1957) merely reducing the quantity of bone marrow injected. This would indicate that the two functions were not independent - rather, that the more urgent requirement for red cells had "drained off" the stem cells which might otherwise have taken part in lymphoid regeneration. The findings here [redacted] that irradiation depleted haemopoietic and lymphopoietic

functions equally, and that foetal cells had these capacities in exactly the same proportions as adult bone marrow cells, are also consistent with a unitary stem cell hypothesis. More direct evidence for the existence of stem cells combining lymphoid and haemopoietic potentialities came from Barnes, Ford, Gray and Loutit (1959) who found a mouse in which the majority of proliferating cells in marrow, thymus and lymph nodes after recovery from irradiation consisted of a karyotypically distinctive clone. A similar clone described here (experiment 16) was also pluripotent, but showed a marked preference for thymus. There is now some evidence that the haemopoietic stem cell function may reside in the marrow lymphocyte (Yoffey, 1960; Cudkowitz, Bennett and Shearer, 1964).

Responses of thymus cells.

(a) Immediately after release from the thymus.

The primary responsiveness of thymus cells has been tested in various GVH situations. They have been found inferior to other lymphoid cells in causing death of the host (Cole and Ellis, 1958; Vos, De Vries, Collenteur and Van Bekkum, 1959; Billingham and Brent, 1959) and in causing splenic enlargement (Cohen, Thorbecke, Hochwald and Jacobson, 1963). Few have attempted a qualitative comparison, but Vos et al, (1959) found that thymus cells were 5% to 50% as active as lymph node cells, depending on the system used. The low activity of thymus cells was confirmed here in GVH splenomegaly (experiment 17) and in transfer of the capacity to make primary antibody responses (experiment 19). In view of the apparent maturation

of thymus cells described in experiment 21 it may be questioned whether some of these cells mature between the times of antigenic challenge and measurement of the response, and that the true potency of thymus cells immediately after release from the organ might not be zero. Two facts can be brought against this. One is that even where the time interval was reduced to two days, as in the mitotic competition experiment (no.18) the thymus cells appeared no less competent than after longer intervals. Another is that after immunisation of an animal with either living tissue (Billingham and Brent, 1959) or protein antigens (Harris, Harris, and Farber, 1954; Dixon, Weigle and Roberts, 1959), thymus cells acquire some ability to transfer secondary responsiveness. The same was seen in experiment 20. It seems very likely that a small proportion of thymus cells are competent in situ, and recent work by Warner (1964) in the chicken suggests that these are to be found among medullary rather than cortical lymphocytes.

(b) Development of competence by thymus cells.

Several attempts have been made to restore responsiveness to thymectomised mice by injections of thymus cells. Where only a few thymus, spleen or lymph node cells were injected shortly after thymectomy, no development of responsiveness (Martinez, Dalmasso and Good, 1964) or protection from wasting disease (Miller, 1963; Parrott and East, 1963) was observed. But where large numbers of thymus cells were injected over a period of weeks after thymectomy the GVH competence of recipient spleens was found to be normal (Yunis, Hilgard, Sjodin, Martinez and Good, 1964). The restoration of

responsiveness to neonatally thymectomised mice by transfer of thymus cells was seen here in experiments 12 and 13, which also indicate that the increase in competence of recipient lymph nodes could be accounted for entirely by the transferred thymus cells themselves, and not by restoration of host-cell competence. The same conclusion was reached by Yunis et al (1964), using Simonsen's discriminant spleen assay. Since the development of full responsiveness took longer after intraperitoneal than intravenous transfer it may be that maturation requires interaction with other cells, perhaps in the spleen, which are reached more quickly by the intravenous route. In any case it is too rapid to be due to multiplication of the 5% or so which was competent originally. No further rise in responsiveness occurred after the first week for thymus cells (or the first day for lymph node cells) and therefore it is unlikely that these cells can increase their numbers in the absence of the thymus. Even in the presence of the thymus it was the cells of bone marrow rather than lymphoid origin which were mainly responsible for repopulating lymph nodes (Ford and Micklem, 1963).

If thymus cells can become competent within a few days of transfer they will presumably do so even after antigenic challenge. The question then arises why the response should be so small when challenge was made immediately after transfer. This was seen after intraperitoneal transfer in the STH, BSA and GVH experiments. Antigen will certainly remain in immunogenic form for sufficiently long in the GVH experiment, and probably also in the BSA experiment. Therefore it is a reasonable hypothesis that most of the thymus cells become paralysed.

The machanism of development of competence may be quite simple. For example it may only require that the cells be removed from the effects of an inhibitory factor present in the thymus. A factor which will inhibit primary immunisation has recently been extracted from serum by Mowbray (1963a,b). Although its concentration was low in serum it was present in large quantities in the thymus, and disappeared from the serum after thymectomy (Mowbray, 1963c).

## PART III: THE FUNCTION OF THE THYMUS IN ADULT LIFE.

### INTRODUCTION.

Thymectomy has been performed in many species of young adult rodents, and has in general been without effect on the growth rate, breeding behaviour, longevity or susceptibility to common infections (Miller, 1963 b). Immunological responsiveness was only slightly depressed (Fichtelius, Laurell and Phillipson, 1961) or not at all (Harris, Rhodes and Stokes, 1948) - a result which stands in contrast to the effects of neonatal thymectomy described in Part II. Yet the thymus does not undergo any noticeable change on the attainment of immunological maturity, and it would be strange if its function should cease at that time. The rate of thymic cell division remains high even in the adult, suggesting that the organ continues to produce lymphoid cells. Since no further growth of lymphoid tissues takes place, it may be that these cells serve to maintain the numbers of lymphocytes against the normal processes of destruction.

The experiments which follow had the primary object of detecting the function of the thymus in adult life, but the results also have implications for the mechanisms of recovery from paralysis and of lymphoid cell turnover. These are discussed later.

METHODS.

Induction of Paralysis.

1. Bovine gamma globulin.

Provided that it is free of any adjuvant material this antigen does not immunise CBA mice, and a single dose is sufficient to paralyse even in adult life (Dresser, 1962). The protein was made up as a 1% solution in saline and then centrifuged at 20,000 g for 20 mins. in order to remove any particulate matter which might act as an adjuvant; and then 0.025ml (containing 250 µg BGG) was injected intraperitoneally. The mice were given sterilized drinking water for 10 days before and after injection in order to minimise the intake of bacteria. At least 14 days were allowed before giving any test challenge, since induction of paralysis takes about 7 - 10 days (D.W.Dresser, personal communication, 1962).

2. Bovine serum albumin.

Even without adjuvant this antigen immunises slightly, and a high degree of paralysis can only be obtained after a prolonged course of injections. Injection schedules were those adopted by Dr. N.A. Mitchison: I. A dose of 250 µg/g body weight, three times per week for 14 weeks starting on the day of birth; II Adult (2 - 3 months old) mice were irradiated with 600r and then injected with 5 mg BSA weekly for 10 weeks.

Antigen elimination.

The rate of elimination of radio-iodine-labelled antigen was measured as a means of assessing the strength of immune

response to BGG. The method was that described by Dresser (1960). The elimination test was made 35 days after immunisation with either BGG Freunds or BGG-AP. Each mouse was injected intraperitoneally with 2 mg of $^{131}$I-BGG, and the elimination of this protein was followed by bleeding at 2 day intervals from the second to the tenth day after injection. The blood was spread on planchettes and the radioactivity counted in a Geiger counter. No correction was made for the loss of antigen by bleeding since this would be the same for experimental and control groups.

Agglutination test for anti-BGG antibody.

Since commercial BGG contains minor immunising components, besides the main paralysing component (Dresser, 1961) the tanned-cell agglutination procedure (Boyden, 1951) cannot be relied on to indicate paralysis. This difficulty has been overcome in the present method, which was developed by Dr. D.W. Dresser from a suggestion by Dr. R. Ceppelini. In principle, mouse red cells are sensitized not with crude BGG but with bovine anti-mouse red cell antibody. This antibody appears not to contain significant quantities of the immunising component because cells sensitized with it were not agglutinated by serum from mice paralysed with BGG. In order to prevent direct agglutination by the bovine antibody the mouse cells were first coated with a Vi antigen ("Vismigen", Instituto Sieroterapico, Milan) before being exposed to the bovine antibody. The action of such bacterial antigens as inhibitors of agglutination is described by Ceppelini and Landy (1963). The procedure was as follows:

Fresh mouse blood was collected in citrate saline. The cells were washed three times in phosphate-buffered saline (PBS) then

made up to 16% and mixed with the same volume of Vi antigen freshly made up to 130 µg/ml in PBS. They were incubated for 15 mins at 37° with occasional agitation, then washed three times in PBS and suspended to 8%. This suspension of Vi-coated cells was then mixed with 2½ times its volume of diluted bovine antiserum. The optimum dilution had been determined by experiment, and was found to be 1/512 for this antiserum. After incubating 30 mins at 37° with occasional agitation, the cells were washed three times and made up to 2.3% in PBS. For titration 0.1 ml of 2.3% sensitised cells was added to 0.2 ml of each dilution of mouse serum. The agglutination patterns were read as described in Part II for SRBC.

## EXPERIMENTS AND RESULTS.

### Effect of thymectomy on recovery from irradiation.

Although irradiation severely depletes lymphoid tissues of their lymphocyte content and depresses primary responsiveness generally, these subsequently undergo almost complete recovery. The object of the following experiments was to determine if the thymus plays a part in the recovery process. In brief, mice thymectomised in adult life were irradiated and then tested subsequently for recovery of responsiveness using either BSA or skin allografts as the antigen.

22. Response to BSA.

(a) Twenty-one male mice were thymectomised as the age of two months, and the same number sham-operated. Two weeks later some of them were irradiated with 600r. They were then divided into three groups, which were challenged with BSA Freunds 2 days

3 weeks and 7 weeks after irradiation. The results, presented in Fig. 17a show that where the sham-operated mice had regained normal responsiveness during 7 weeks, the thymectomised mice had not recovered at all. No significant difference was found between thymectomised and sham-operated mice in the non-irradiated groups.

(b) The same general result was obtained in a similar experiment. This time the mice were females, and while some received 600r, others received 900r + bone marrow. Challenge was with BSA-AP (Fig.17b).

23. Allograft rejection.

The mice were females thymectomised or non-operated at 2 months and given 900r + bone marrow at 3 months of age. They all received BSA Freunds shortly after irradiation, as part of another experiment. Five months after irradiation each received a graft of C57BL tail skin. The rejection times are given in Table 17. Three grafts failed to take - perhaps because of the general debility of the mice as a result of irradiation. Although three grafts in the thymectomised group were rejected within the normal time, the remaining seven showed greatly prolonged survival.

Effect of thymectomy on recovery from paralysis.

The principle of these experiments was the same as the last, except that the depression of immune function was specific rather than general and therefore might be expected to leave intact whatever processes of lymphoid cell turnover remain after thymectomy.

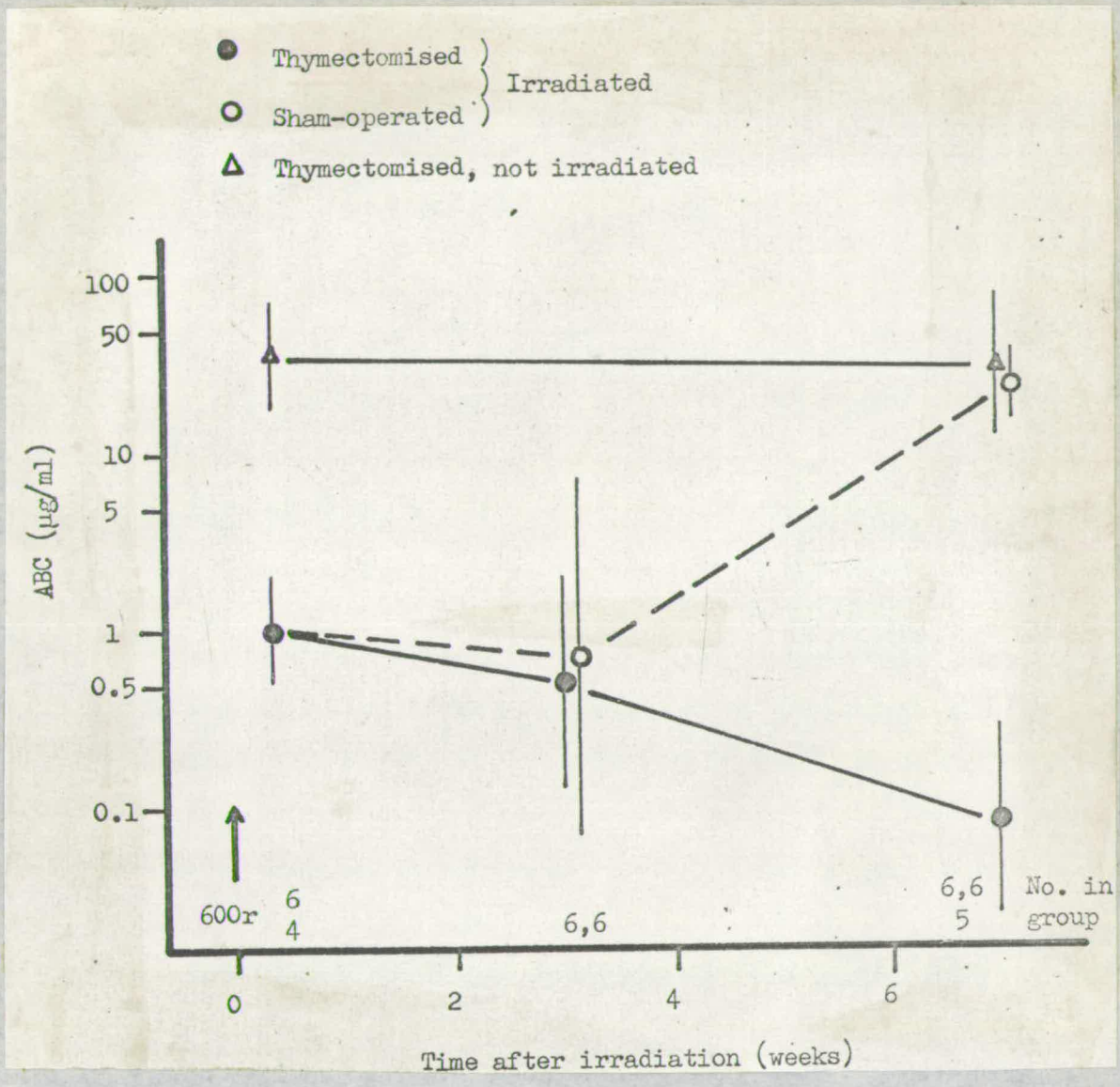


**Fig. 17(a). Recovery of antibody response after irradiation.**

**Male mice thymectomised at 2 months old, irradiated with 600 r, and challenged with BSA Freund's at intervals after irradiation. They were bled 40 days after challenge.**

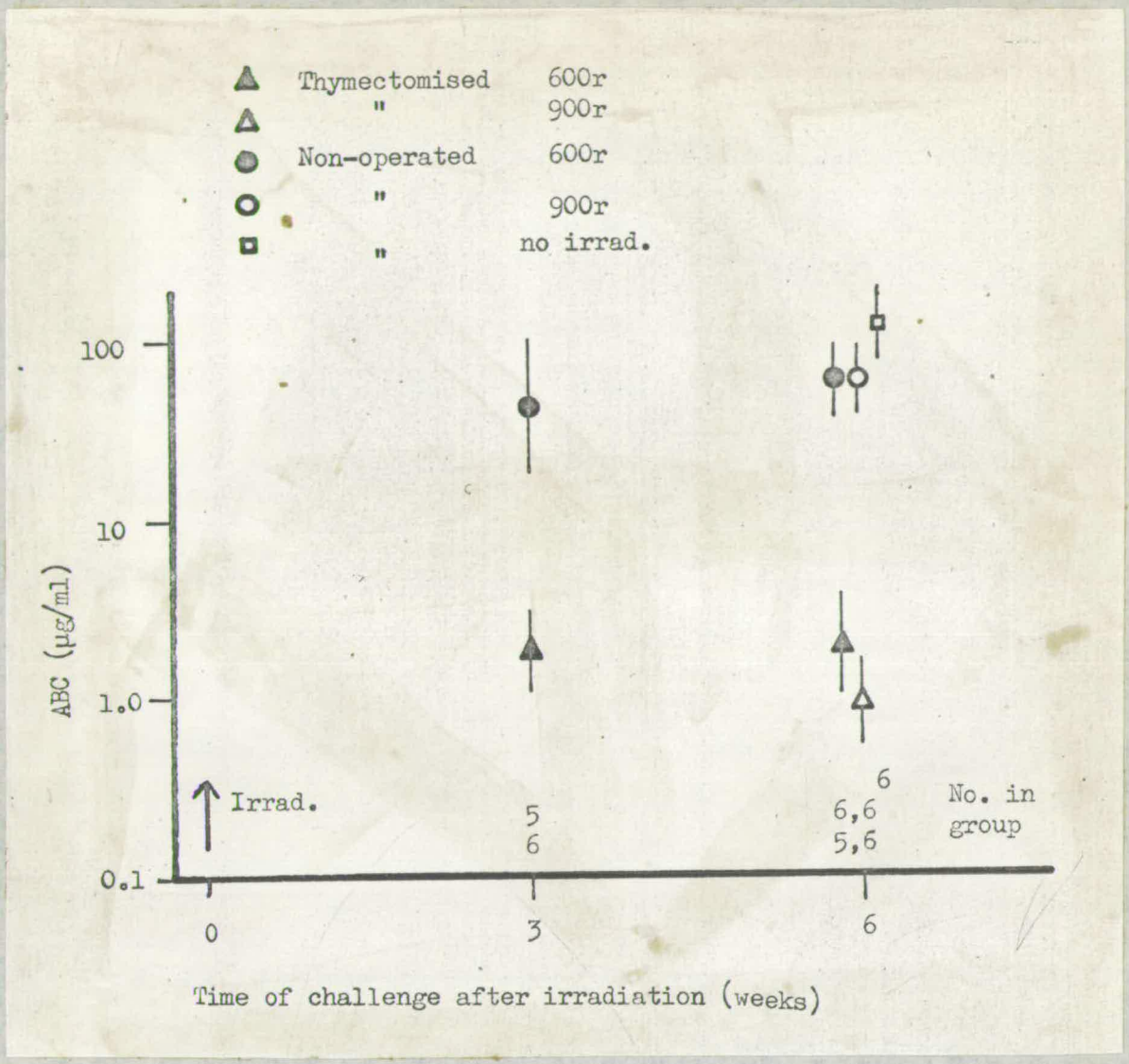


**Fig. 17(b). Recovery of antibody response after irradiation.**

**This experiment is similar to that shown in Fig. 17(a) except that the mice were females, and some received 900 r rather than 600 r, and they were bled at 28 days after challenge with BSA-AP.**

Table 17. Effect of thymectomy on recovery of the capacity to reject skin allografts after irradiation.

| | Day after grafting : Mouse died with graft intact | Graft completely rejected |
|---|---|---|
| Thymectomised | 42,42,86,91,119. | 10,12,14,36, > 119. |
| Non-operated | | 11,11,13,13,13,14. |

Two grafts on thymectomised and one on non-operated mice were technical failures.

24. Paralysis to BSA.

Male mice were paralysed according to injection schedule I. Half of them were thymectomised three weeks after the last paralysing injection. Groups consisting of six thymectomised and six non-operated mice were then challenged at intervals up to 24 weeks after thymectomy, with BSA Freunds. In addition one control group of the same age, neither paralysed nor thymectomised, was challenged at 12 weeks. The results are shown in Fig.18. The non-thymectomised mice were beginning to lose paralysis at 4 weeks and had regained normal responsiveness by 12 weeks after thymectomy. By contrast the recovery in the thymectomised mice was slower, and had ceased by 12 weeks, the antibody titres remaining thereafter at about 3% of control levels. The specificity of paralysis was then tested in three of the groups (17,20 and 24 weeks groups) by challenge with sheep red cells. The responses, which were assessed by agglutination, were not significantly different as between thymectomised and control mice (Table 18).

25. Paralysis to BGG.

Forty-eight 3 month old male mice were injected with 250 µg centrifuged BGG and 32 left non-paralysed as controls. Half of the paralysed and half the control mice were thymectomised 12 - 15 days after this injection. Equal numbers of thymectomised and control mice, among the paralysed group, were challenged at 3, 12 and 24 weeks after the paralysing injection, while the non-paralysed mice were challenged at 3 and 24 weeks only. The first challenge with BGG Freunds resulted in erratic responses in the

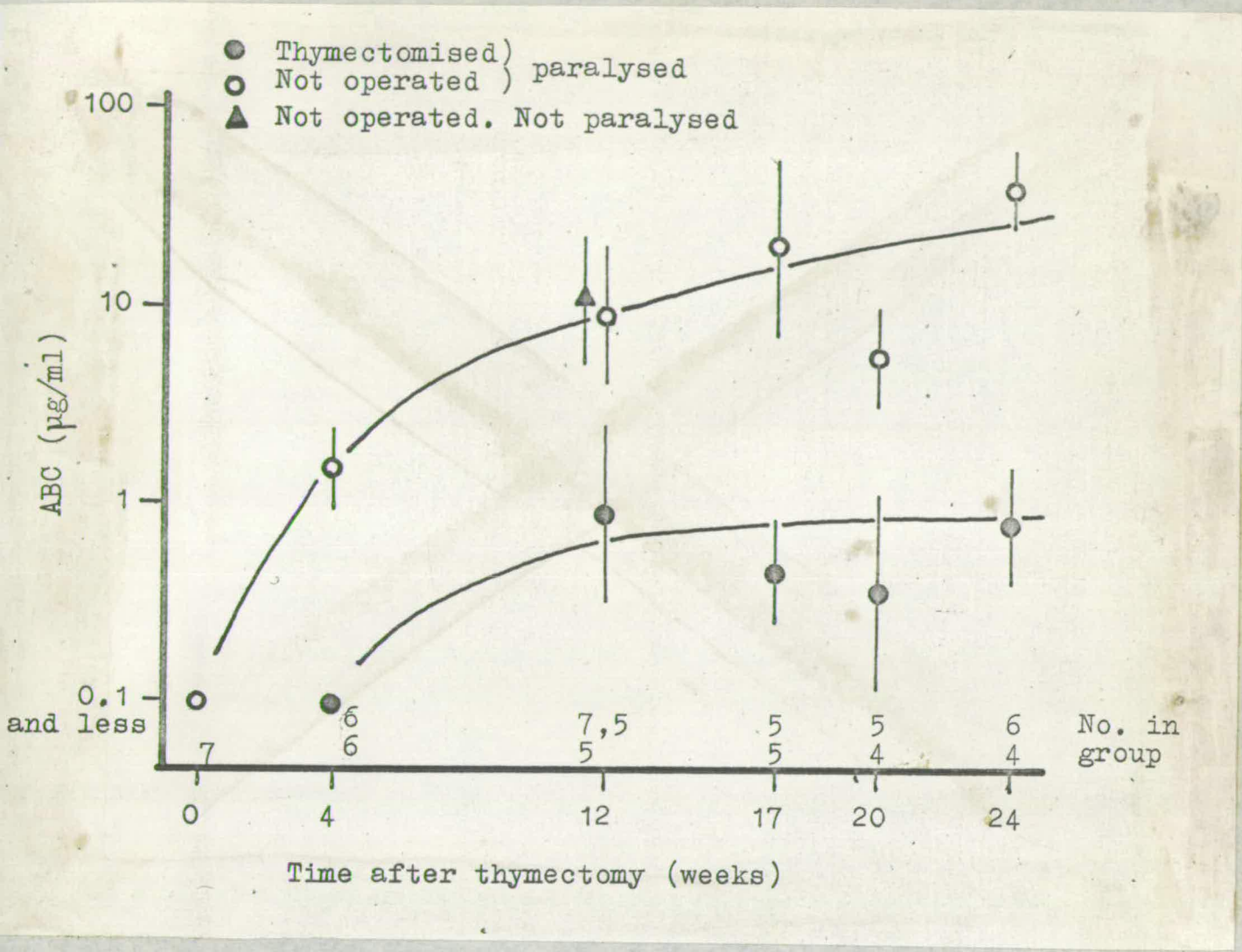


**Fig. 18.** This diagram illustrates the effect of thymectomy in retarding recovery from paralysis to BSA. Groups of mice were challenged with BSA Freund's at intervals after thymectomy and bled at 20 days after challenge.

Table 18. Anti-sheep red cell agglutinin production by thymectomised and non-operated mice which had been paralysed to BSA. They were challenged with sheep red cells 30 weeks after thymectomy. The experiment shows that although the thymectomised mice failed to recover properly from paralysis to BSA (see Fig.18) their responsiveness to an unrelated antigen was little depressed.

| | No. of mice producing serum agglutinating to the following titres: ($\log_2$) | | | | |
|---|---|---|---|---|---|
| | 7 | 8 | 9 | 10 | 11 |
| Thymectomised | 1 | 1 | 5 | 4 | 2 |
| Not operated | - | - | 4 | 8 | 4 |

non-paralysed controls. For this reason BGG-AP was used in the groups challenged subsequently. The mice of the 24 week group were bled again 21 days after the injection of $^{131}$I-BGG and the sera titrated for specific antibody by the Vi agglutination test. The results of the eliminations (Fig.19) show that thymectomy has inhibited the recovery from paralysis, but the effect is less striking than in the BSA experiment. However, the eliminations at 24 weeks are so rapid as to approach the limits of the useful range of this test, and the results of the agglutination (Table 19) probably give a better quantitative comparison. They emphasize the fact that recovery from BGG paralysis, even with the thymus intact, was far from complete at 24 weeks. The responses of the non-paralysed controls show a very slight depression of the response resulting from thymectomy alone (Fig. 19 a and c). The substance of experiments 24 and 25 has already been published (Taylor, 1964).

Effect of transfer to irradiated recipients on the state of paralysis in dissociated cells.

During recovery from paralysis cells reappear which are capable of responding to the antigen used for paralysis. These may arise either from stem cells or from mature cells already competent but specifically paralysed. Since the thymus is concerned with the maturation of new competent cells the effect of thymectomy just noted would indicate that stem cells are likely to be the major source in recovery from paralysis. However, some recovery was observed even in the absence of the thymus. The following experiment is an attempt to answer the question of whether this recovery also was dependent on lymphoid

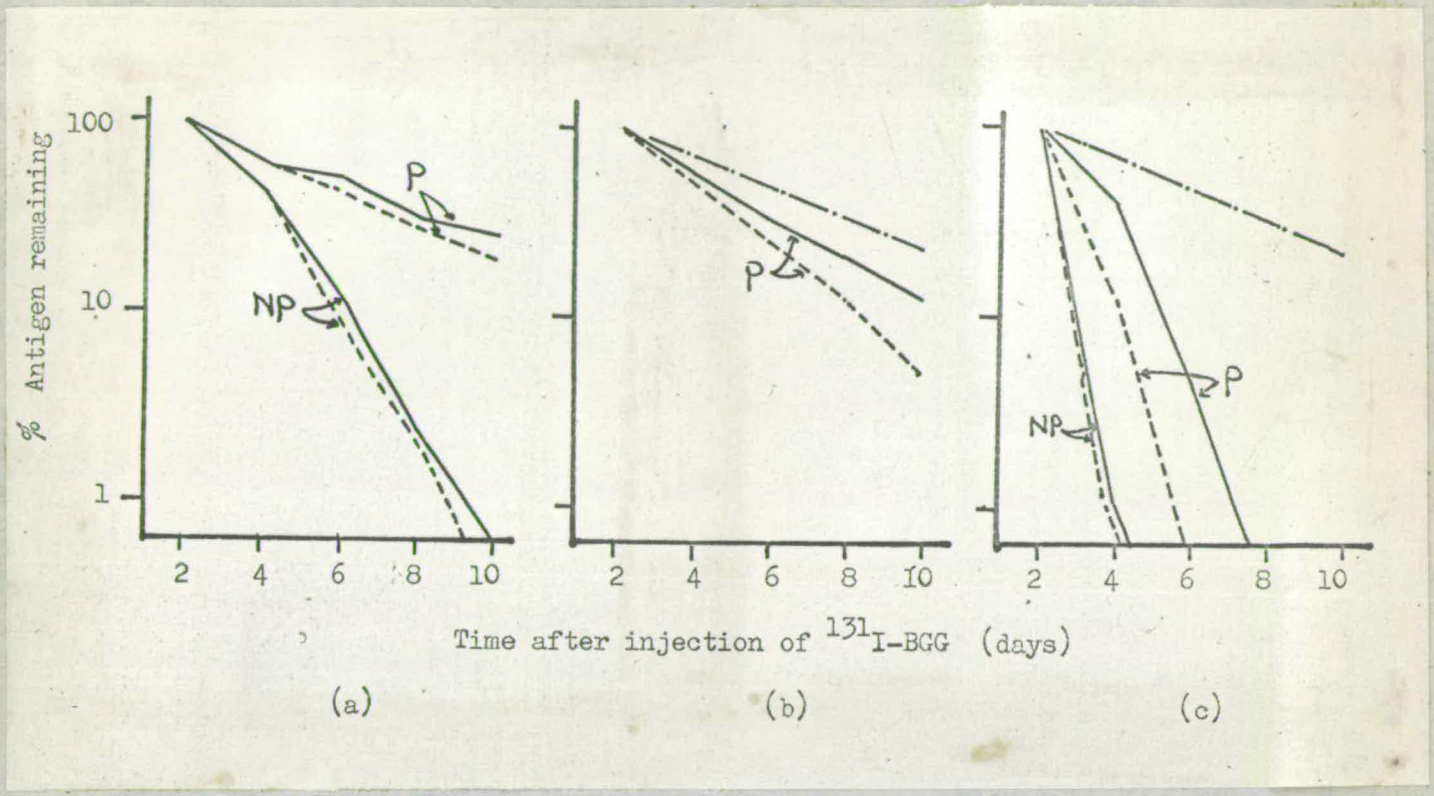


Fig. 19. Effect of thymectomy on recovery from paralysis to BGG. Each line represents the mean elimination response for a group of 5 - 8 mice. The graphs show the results of challenge at 3 weeks (a), 12 weeks (b) and 24 weeks (c) after thymectomy.

_____ Thymectomised groups.

_ _ _ Sham-operated groups.

_ · _ Mean rate of elimination in fully paralysed mice for comparison.

P = Paralysed.

NP = Not paralysed.

Table 19. Serum anti-BGG antibody titres from the group of mice challenged 24 weeks after thymectomy (see Fig.19).

| | | No. of mice producing serum agglutinating to the following $\log_2$ titres: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | <3 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Paralysed | Tx | 3 | 4 | 1 | - | - | - | - | - | - |
| | Sh | - | - | - | 2 | 1 | 2 | 1 | 1 | - |
| Not Paralysed | Tx | - | - | - | - | - | - | [illegible][1] | 3 | 1 |
| | Sh | - | - | - | - | - | - | 3 | - | 3 |

Tx = thymectomised
Sh = sham-operated

cell turnover. Since irradiation destroys lymphoid tissue it was thought that paralysed lymphoid cells transferred to irradiated-thymectomised recipients might be stimulated to proliferate in compensation; and that if recovery from paralysis depended on cell turnover, then it should be accelerated by this procedure. The experiment also offered an opportunity to test the state of paralysis in thymus cells taken from paralysed donors.

26. Donor mice were males and were paralysed with BSA by schedule II. They were killed 10 days after the last paralysing injection at the age of 5 months and suspensions made of their lymph node and thymus cells. Similar suspensions were made from an equal number of untreated donors. The recipients were thymectomised females irradiated with 600r. Groups of them received either one donor-equivalent of lymph node cells or four donor-equivalents of thymus cells, or no transfer. The thymus-cell recipients were challenged 3 weeks later in order to allow time for the development of full competence by these cells. The other mice were divided into groups and challenged 24 hours, 3 weeks and 7 weeks after the cell transfer. In addition, some of the paralysed donors were challenged at 3 and 7 weeks. The results are shown in Fig. 20. Unfortunately, due to the low dose of irradiation given to the recipients, their responses after receiving normal lymph node cells were not always significantly higher than they were without transfer. However, there is no indication of recovery by the paralysed lymph node cells, although the paralysed donors (with thymus intact) made a

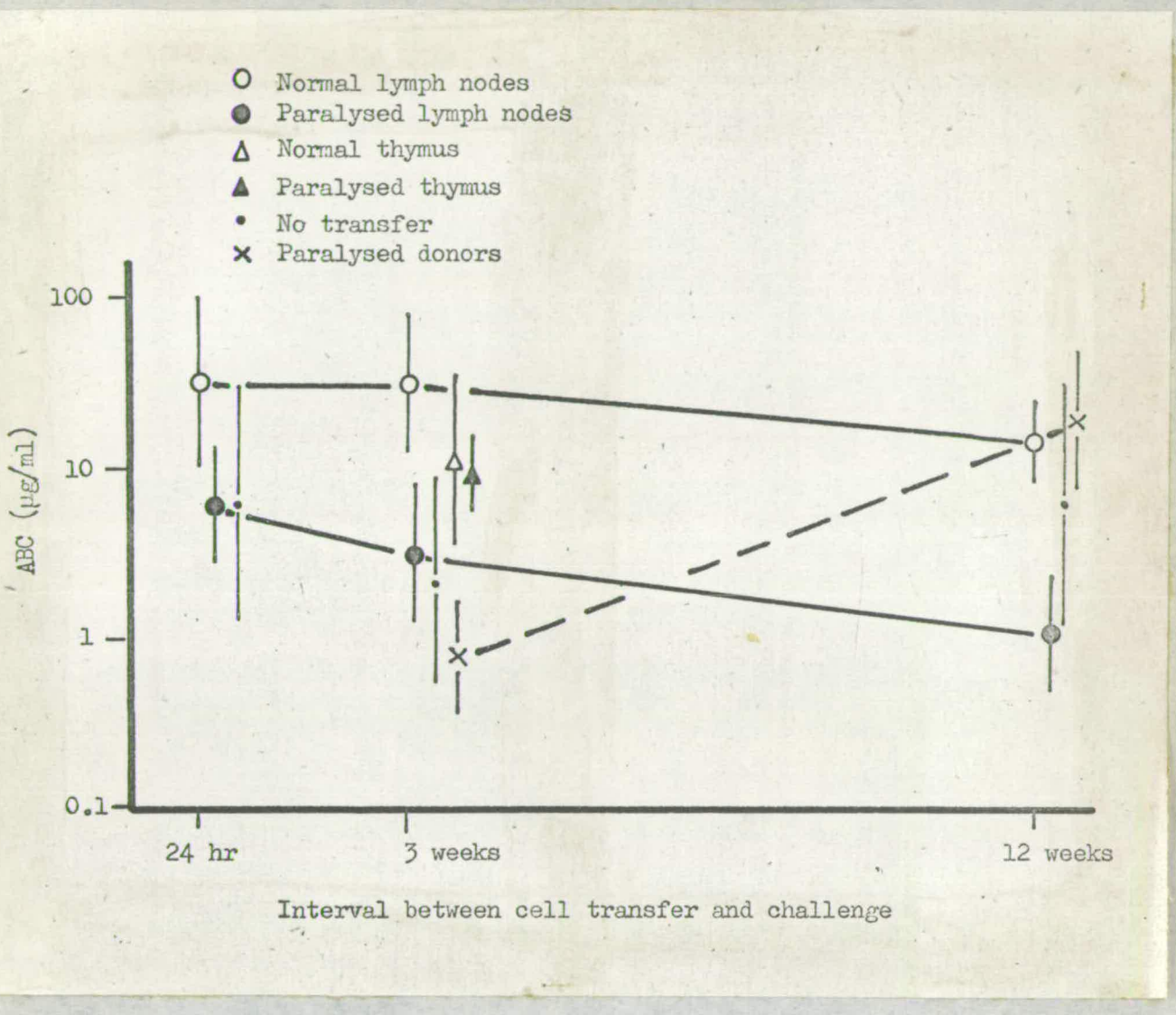


Fig. 20. Anti-BSA antibody production by lymph node and thymus cells taken from normal and specifically-paralysed donor mice and transferred to irradiated-thymectomised recipients. The diagram illustrates the failure of lymph node cells to recover from paralysis under these conditions, and the lack of effect of paralysis on the responses due to thymus cells.

significant recovery. The transfer of paralysed cells to an irradiated environment, therefore, failed to accelerate their recovery from paralysis. Indeed, it failed equally to stimulate proliferation, as judged by the constancy with time of the responses of mice receiving normal lymph node cells. In the thymus cells on the other hand no effect of paralysis was detectable.

Decay of responsiveness after thymectomy.

The object of these experiments was to compare the responsiveness of mice thymectomised in adult life against that of non-thymectomised controls at intervals after thymectomy in order to determine whether the thymectomised mice tended to lose their responsiveness.

27. GVH spleen weight assay.

Female mice were thymectomised or sham-operated at 8 weeks old. Groups of 8 - 16 mice were tested by GVH assay at various intervals up to 52 weeks after thymectomy. In each assay the mixed lymph node and spleen cells of one thymectomised mouse were compared for immune potency against those from one sham-operated control as described in Part I. At 52 weeks the potency of the controls might be expected to have declined somewhat through age alone (Makinodan and Peterson, 1962), therefore both thymectomised and sham-operated mice were assayed against normal controls aged 4 - 6 months. The results of the assays are shown in Table 20. and the decay of responsiveness represented graphically in Fig.21. The responsiveness of thymectomised mice remained level with controls for about 25 weeks then dropped

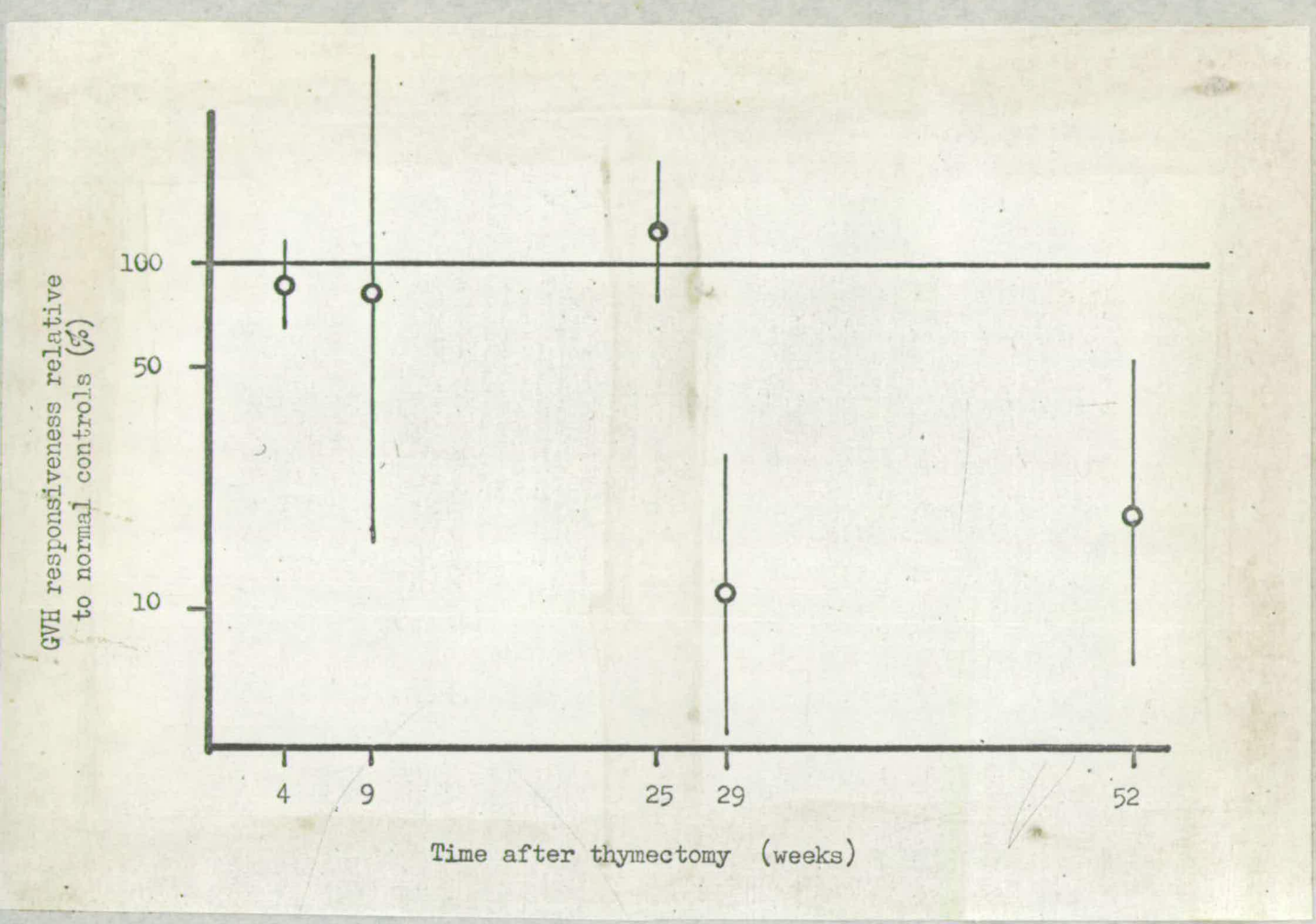


Fig. 21. Decay of GVH responsiveness after thymectomy in adult life. The results from Table 20 are here shown in graphical form.

Table 20. Decay of responsiveness. Results of GVH spleen weight assays using mixed lymph node and spleen cells. Thymectomised mice were compared with sham-operated controls of the same age in the 4, 9, 25 and 29 week groups. In the 52 week group the thymectomised and sham-operated controls werecompared with normal mice of about 5 months.

| Time after operation | No. of assays | Relative potency | Relative cell number | Relative responsiveness |
|---|---|---|---|---|
| | | ( Geometric Means ) | | |
| 4 weeks | 6 | 0.97 | 0.89 | 0.86 |
| 9 weeks | 5 | 0.84 | 0.99 | 0.83 |
| 25 weeks | 3 | 1.61 | 0.79 | 1.27 |
| 29 weeks | 3 | 0.21 | 0.54 | 0.12 |
| 52 weeks | ( 15 | 0.24 | 0.82 | 0.20 |
| | ( 9 , | 0.64 | 1.12 | 0.72 |

P < 0.01 for difference between 25 and 29 week groups.

P < 0.001 " " " Tx and Sh at 52 weeks.

sharply and remained thereafter at about 15% of normal. The decline of responsiveness in sham-operated controls by 52 weeks was only slight. While the high variability lay partly in the technique, it could be seen where repeat assays were done on the same cell suspensions, that much of the variability in the 52 week group was real.

28. Challenge with BSA.

The results presented below have been collated from four experiments set up with other primary objects in mind. The mice were thymectomised either at 4 weeks old or at 10 - 14 weeks old. At various times afterwards thymectomised and control groups were challenged with BSA Freunds and the serum antibody titrated at 20 and 40 days later. Since the absolute titres varied considerably from one experiment to another (probably due in part to variations in the efficacy of Freunds adjuvant) the titres from the thymectomised mice have been presented as a percentage of the controls in each group (Fig.22a). The decay of responsiveness is much less marked than in the GVH assay experiment, but as mentioned in Part I a small difference in antibody titre may reflect a large difference in the number of competent cells. However, the general pattern was the same in that the rate of decline of responsiveness tended to increase with time after thymectomy. The apparent initial fall in those thymectomised at 4 weeks old reflects an <u>increase</u> in the responsiveness of the controls due to normal growth processes rather than a <u>decrease</u> in the thymectomised mice. In Fig. 22b the results from 20 and 40 day bleedings have been plotted separately in order to reveal

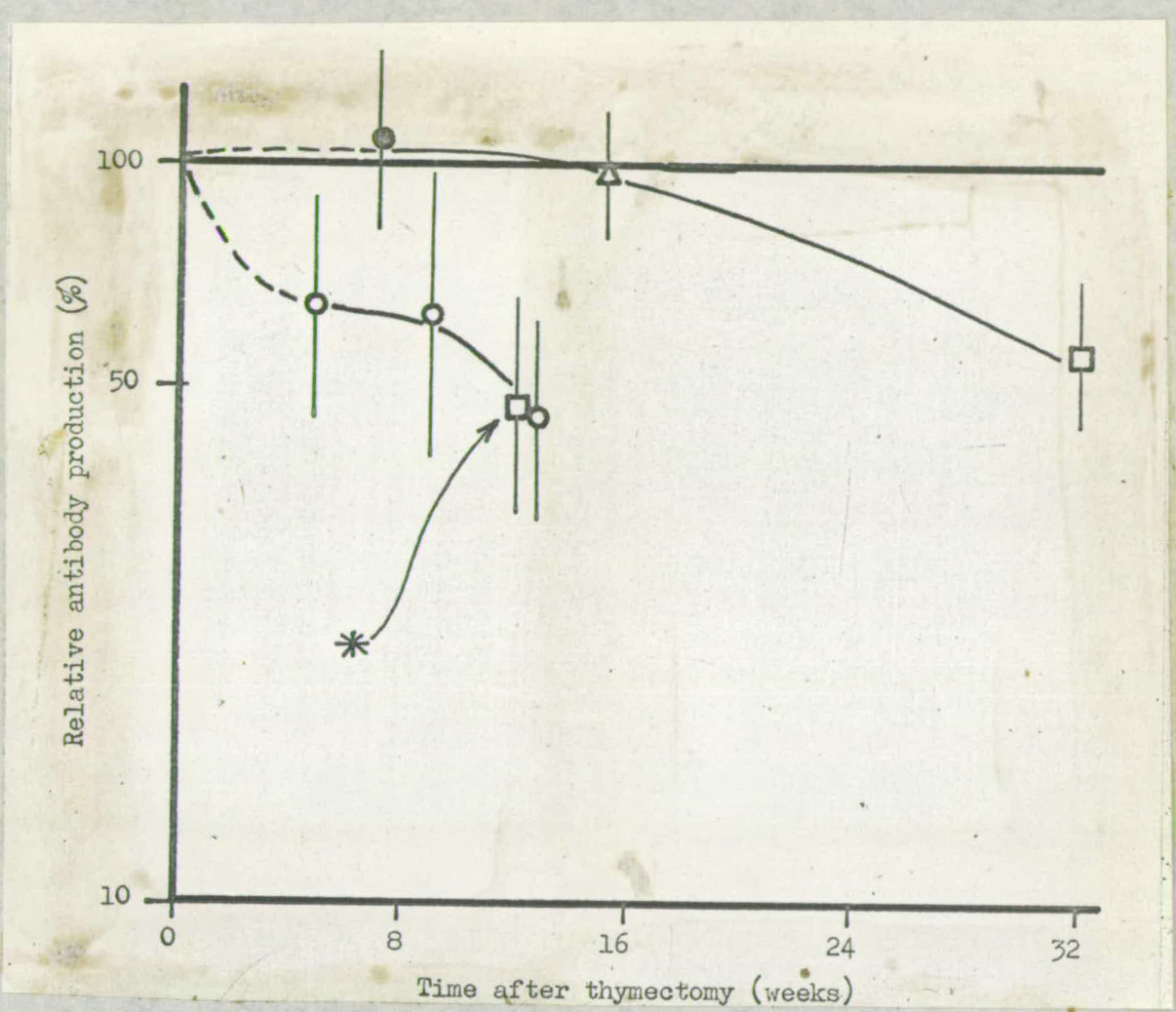


Fig. 22(a). Decay of responsiveness to BSA after thymectomy in adult life. Results have been taken from 4 separate experiments (represented by different symbols), and expressed as:

$$\frac{\text{Mean titre in thymectomised}}{\text{Mean titre in control}} \times 100,$$ for each group. The results from 20 and 40 day bleedings have been combined.

Mice in the experiment denoted by open circles were thymectomised at 4 weeks old; the rest at 10 - 14 weeks.

Asterisk indicates a point falling out of line with the indicated trends.

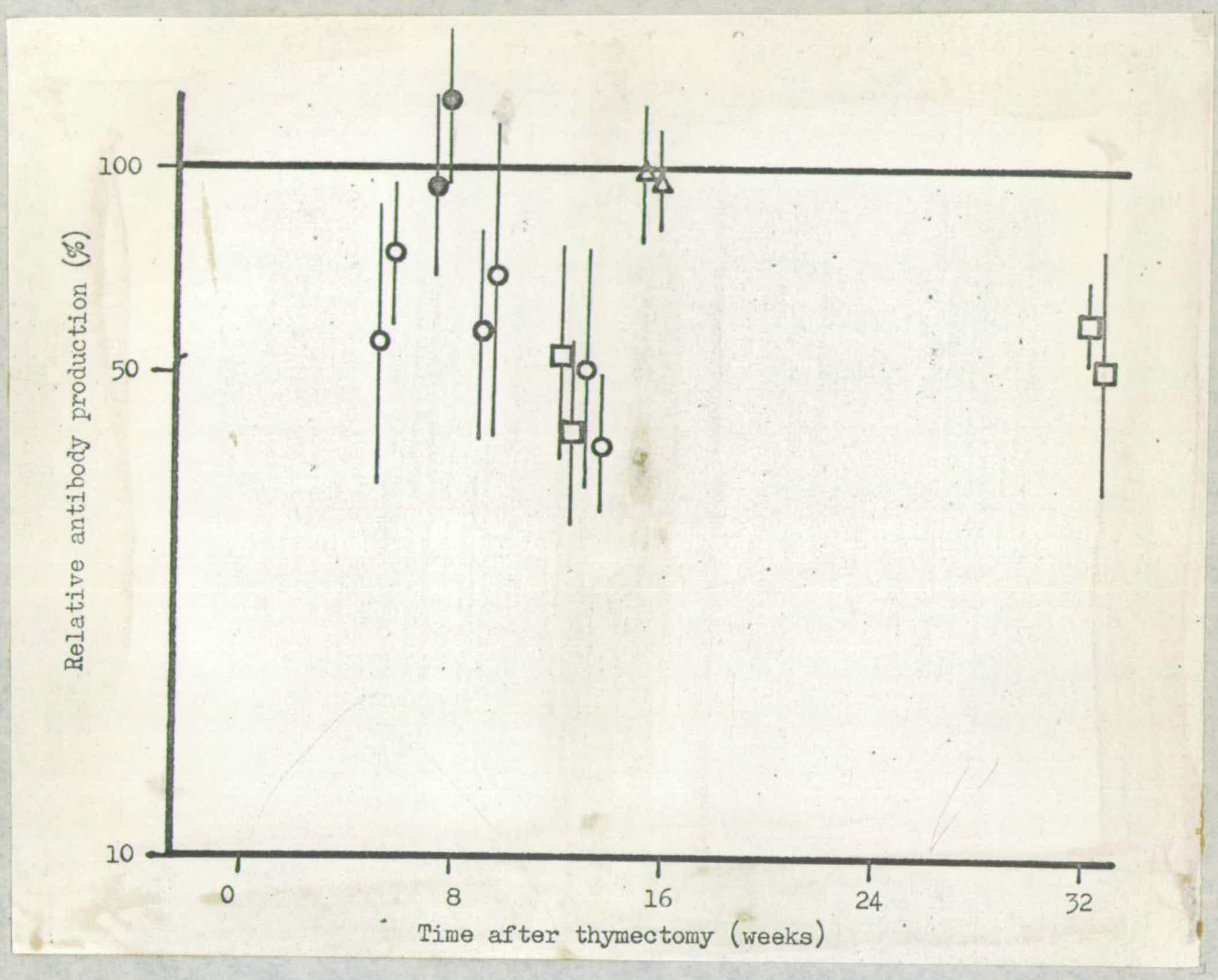


Fig. 22(b). This diagram is identical with Fig. 22(a), except that the results from 20 and 40 day bleedings are plotted separately, and are here represented by the left- and right-hand symbols of each pair respectively.

another feature: when challenged soon after thymectomy the effect of the operation is most evident at the 20 day bleed, but the reverse is seen in mice challenged at longer intervals after thymectomy.

Wasting Disease after adult thymectomy.

It was noticed in the mice used for experiment 27, at 29 weeks after thymectomy, that all the thymectomised groups exhibited a ruffled condition of the fur (Fig.23), while the non-thymectomised controls had normal smooth fur. This condition had not been seen in these mice at 25 weeks - although as it was not expected it might have been passed over. An examination was then made of all other mice thymectomised in adult life, and was found to be present in two groups 30 and 41 weeks after thymectomy, but absent in one group at 9 weeks. The 30 week group (which had been immunised with BGG-AP 8 weeks previously, as part of experiment 25 were killed and examined grossly. The lymphoid organs were weighed, and histological sections made of some of the lymph nodes and skins. The results of weighing are shown in Table 21. Although the body weight was slightly less in the thymectomised mice, the lymph nodes were nearly twice as large as they were in controls. Spleens on the other hand were rather smaller. The thymectomised mice had very little sub-cutaneous fat. Their lymph nodes had a relative deficiency of small lymphocytes, but plasma cells appeared to be abundant. Their skin was abnormally thin both in the dermis and epidermis - indeed the latter was often only two cells thick.

The causes of these symptoms are obscure. They do not

Fig. 23. "Wasting disease" after thymectomy in adult life.

One mouse (left) was thymectomised and the other sham-operated at 12 weeks old. The difference in fur condition 30 weeks later is evident.

Table 21. "Wasting disease" in mice thymectomised in adult life: effect on body and organ weights. The mice were thymectomised at 14 weeks and killed at 44 weeks old.

| | No. in group | Body weight (g) | Lymph node weight (mg) | Spleen weight (mg) |
|---|---|---|---|---|
| Thymectomised | 8 | 19.5 ± 3.5* | 57 ± 10 | 64 ± 6 |
| Sham-operated | 8 | 22.2 ± 4.4 | 33 ± 5 | 72 ± 9 |

*Standard deviations

seem to be specifically related to thymectomy since the features of ruffled fur and enlarged lymph nodes were often seen in very old mice (usually over 18 months), and once in a litter at weaning. A reasonable interpretation would be that the cause is an infectious agent which can attack mice more readily after thymectomy.

## DISCUSSION.

### Evidence that the thymus continues to function in adult life.

The effect of thymectomy on the recovery of immune responsiveness after irradiation has been described by Miller (1962b) and Miller, Doak and Cross (1963) using sheep red cells or skin allografts as the antigen. It is clear from their work, and from the foregoing experiments (nos. 22 and 23) that the thymus is necessary for the restoration of immune function after radiation damage. If there were still any doubt that the thymus has a part to play in adult life, this was dispelled by the demonstrations, first that the thymus is necessary for proper recovery from paralysis to protein antigens (expts. 24 and 25), and second that it is necessary for the maintainance of immune responsiveness to its normal level (expts. 27 and 28).

### Decay of responsiveness.

It is very probable that in adult life the thymus continues to bring about the production of competent cells from stem cells by the same mechanisms which have been considered for the neonatal thymus in Part II, and in this way maintains the numbers of competent cells in the body. The actual course of the decay

curves, is not so easily explained. If the effect of adult thymectomy was to cut off entirely the supply of new competent cells, then the ensueing decay of responsiveness should reflect the rate of loss of the competent cells already present. Their numbers might be expected to decay in linear fashion if they had a definate life span, or exponentially if their life depended on chance events - such as encounter with antigens. Neither expectation was borne out in the decay curves (Figs. 21 and 22), where the rate of decay actually tended first to increase with time after thymectomy, and then (at least in the GVH experiment) to cease so that responsiveness remained constant at a lower level. Although the variability of individual determinations was high in the GVH experiment there can be little doubt that an inflection in the curve occurred at about 27 weeks after thymectomy (Fig.21.) While being less dramatic, an acceleration in the decline of responsiveness probably occurred in the BSA experiments also (Fig.22). Two other phenomena, occurring about the same time as this inflection emphasize its reality as a fairly abrupt event. One of these is seen in Fig.22b, in which antibody titres are plotted separately for bleedings 20 and 40 days after challenge. In those mice challenged before the inflection, thymectomy has depressed the 20 day more than the 40 day titres, whereas the reverse effect can be seen in those challenged after the inflection. The other phenomenon was the change in coat and general condition which has already been described. Although there was a distinct fall in immune responsiveness it never reached the levels seen after neonatal thymectomy or irradiation, but appeared to attain equilibrium at

some 15% of the normal value.

The course taken by the decay of responsiveness curves cannot thus be explained by the simple hypothesis that the thymus controls all competent cell production. It is open to many interpretations, and more work is required before a clear picture can emerge. However, it was felt that a working hypothesis, which would also account for the paralysis results, would be valuable at this stage.

Precursor cell hypothesis.

It is proposed that, as a result of thymus action in early life, a pool of precursor cells is built up in the body. These cells would not themselves be competent, but would be capable of transformation to competent cells without further dependence on thymus action. In the decay of responsiveness experiments the numbers of competent cells would be maintained initially to a homoeostatic level by transformation of precursors, until eventually the stock of the latter would become exhausted and cause the sharp fall in responsiveness which was observed.

The fact that a few lymphoid cells can make a disproportionately large response after transfer to irradiated recipients (Makinodan, et al, 1962; and experiments 19a and b in the present work) might also be brought to book. This could be due to an enhanced multiplication of committed cells after they have been stimulated by antigen, as is often assumed (e.g. by Dixon and McConahey, 1963); but it could equally be due to an increase in the number of competent cells before antigenic stimulation. A corollary to this would be that the precursors must, like

competent cells, be able to survive the manipulations involved in cell transfer.

There are other reasons for believing that only a small proportion of lymphocytes are competent at any one time. In the first place it is well known that a response to one antigen does not inhibit the response to another antigen - although such "antigen competition" effects can sometimes be obtained if the antigens are administered in close succession. This may indicate merely that during their recirculation lymphocytes spend most of their time in situations inaccessible to antigen. On the other hand Gowans (1962) found that only a very small proportion of injected lymphocytes transformed into large pyroninophilic cells during a GVH reaction, and Hirschhorn (1964, unpublished) has found the same in lymphocyte anti-lymphocyte reactions in vitro.

It will be an advantage at this point to summarise the properties required of precursor cells:

(a) Ability to transform into competent cells, and in doing so to maintain the numbers of the latter to a homoestatic level. This function would be independent of the thymus.

(b) Ability to survive cell-transfer.

(c) Radiosensitivity. This would account for the permanent abolition of responsiveness by irradiation after thymectomy.

(d) Inability to increase or replenish their own numbers - at least without thymus action (see below).

(e) Ability to carry the property of paralysis (see below).

It is tempting to equate the properties of thymus cells with those required of precursors. Thymus cells were found

earlier to be capable of transformation into competent cells. They are radiosensitive, survive cell transfer, and probably do not proliferate in the absence of the thymus. It remains to be seen whether they can carry the property of paralysis. Although a preliminary experiment suggested that they did not acquire paralysis while in the thymus, this may only reflect a failure of antigen to enter the thymus, and it seems likely that they would be able to do so after release from the thymus (see Part II).

Production of competent cells in the absence of the thymus.

Although the major source of competent cells appears to be from stem cells by way of a thymus-dependent process of maturation, it should be considered whether a slow rate of production might occur in the absence of the thymus. It is clear first that no significant production occurs after neonatal thymectomy, or after adult thymectomy followed by irradiation - even though lymphoid stem cells are present in adequate numbers in the bone marrow (Miller et al, 1963). Then normal lymph node cells evidently cannot proliferate in such a way as to bring about a net increase in competent cells (experiments 21b and 26) - indeed the failure of such cells to break from paralysis after transfer suggests that there was no turnover of competent cells at all. Nevertheless, there might still exist a mechanism of lymphopoiesis which differed from the thymus-dependent type in being more radiosensitive, and which was present in mature intact lymphoid tissue but absent in the neonate. A basal mechanism of lymphopoiesis such as this could explain the persistent low level of responsiveness in experiment 27 which lasted from 29 weeks to the end of the experiment at 52 weeks after thymectomy.

An alternative would be that there exists a sub-population of competent cells which have a very long life. The first hypothesis is more attractive because it could also explain the limited recovery from paralysis which occurred after thymectomy (see below).

Recovery from paralysis.

The effect of thymectomy on recovery from paralysis to BGG has also been observed by Claman and Talmage (1963) whose results closely parallel the BGG elimination data described here. The results of both experiments (nos. 24 and 25) indicate that although recovery from paralysis is very largely prevented by thymectomy, there was nevertheless a significant degree of recovery even in the thymectomised animals. In the BSA experiment recovery was not only retarded after thymectomy, but it appeared to cease altogether at about the time that recovery in the intact mice became complete. The same could not be seen in the BGG experiment, but here the tempo of recovery in both thymectomised and control groups was much slower and recovery was far from complete even at 24 weeks after the paralysing injection.

Recovery might take place in the following ways (Medawar, 1960) :-

(a) Mature paralysed cells lose antigen from some controlling site without undergoing cell division.

(b) Mature paralysed cells divide and in doing so dilute out the paralysis mechanism.

(c) New competent cells arise from stem cells; while mature paralysed cells either do not exist (if paralysis mean selective

destruction of specifically reactive clones of cells: Burnet, 1959) or else are incapable of recovery.

Most evidence suggests that cell turnover is important, as in hypotheses (b) and (c). For example, the dependence of the rate of recovery on the age of the animal (Mitchison, 1962;1963) and the hastening of recovery by irradiation (Mäkelä and Nossal, 1962) but the occurrence of (a) cannot be eliminated. It was seen in Part II that the main source of increase of competent cells during growth is likely to be from stem cells by a thymus-dependent process of maturation, and therefore the fact that recovery from paralysis was so largely dependent on the thymus would argue that the stem cell mechanism (c) is the principal one. However, the occurrence of limited recovery even after thymectomy invites other explanations.

One possibility is that the operation of thymectomy might not remove all the thymus-like tissue in the body. But the failure to observe any recovery after neonatal thymectomy, or after adult thymectomy with irradiation, argues against this. Another is that recovery might occur by the transformation of precursor cells to competent cells which has just been proposed. But the rate of decay of responsiveness between 25 and 29 weeks in experiment 27 suggests that competent cells turn over fairly quickly - certainly fast enough that the majority would have been turned over during the 24 weeks of the paralysis experiments, yet the actual recovery was very slight. Moreover, it has been argued previously that the disproportionate response of small numbers of cells after transfer to irradiated recipients is due

(at least in part) to cells which were not competent before the transfer; and yet cells from a paralysed mouse failed to recover in the same situation. If the above arguments are valid, they would further imply that precursor cells can carry the property of paralysis.

Any hypothesis for the recovery after thymectomy must explain why this should cease at about 12 weeks after thymectomy in the BSA experiment. This observation might best be accomodated by a third possibility. This is that there exists a basal lymphopoiesis in lymphoid tissues after adult thymectomy - such as has already been invoked to explain the basal level of responsiveness in the decay experiments. This mechanism would maintain the number of competent cells at a subnormal level, but would not be able to build up the full complement of these cells.

CONCLUSION

It is now clear that the thymus plays an essential part in the production of competent cells. It is also clear that at least a substantial proportion of these cells start in the adult as stem cells, identical to those which serve haematogenous pathways of differentiation. They then enter the thymus and therein undergo some necessary change before they can become competent. It may now be asked what is the nature of this change and what bearing it has on general problems of immunity. The answer will depend on whether a competent cell is multipotent for the antigens to which it can react, or whether its competence is restricted to any extent. In the first case all competent cells could be identical; and so their maturation need not differ basically from the inductive processes involved in differentiation of any other cell types. The inductive factor in the thymus would then serve simply to "switch on" the genes controlling immunoglobulin production, by a process which might be compared with the step in red cell maturation which requires induction by erythropoietin.

If competence is much restricted on the other hand, the known mechanisms of differentiation would strain at the number of cell types required. One could conceive of a complex scheme of epigenesis in the thymus, but this would surely require a correspondingly complex and definite

morphology - and such an "immunological homunculus" is certainly not evident in the thymus. Or else one can reject defined mechanisms of differentation, and suppose instead that competent cells become diversified by a process of random mutation at some locus concerned with immune specificity (Burnet, 1959). According to this theory, only immature cells would have the necessary high rate of mutation; and they would have the further property of being particularly susceptible to destruction by the effects of antigen-antibody reactions at the cell surface. In this way the potentially auto-immune mutants would be eliminated.

This process of mutation and selection has not yet been further defined, but some more recent speculations would site it in the thymus (Burnet, 1962; Miller, Marshall and White, 1962). Here there would presumably be selection against auto-immune clones, but not yet selection for any particular foreign antigens. Therefore if the mutations are to be purely random it would be remarkable if there were not some discrepancies in the reactivity to different antigens between thymus and lymph node cells. For if thymus cells are indeed insulated to any extent from contact with exogenous antigens they will tend to exhibit the specificities resulting from mutation alone, whereas among lymph node cells the competing clones will have had opportunity to wax or dwindle according to their antigenic experiences. The demonstration in Part II that thymus cells will attain par with lymph node cells within a few days of transfer offers a means of searching for such discrepancies.

None was found here with regard either to BSA or to GVH responses against C57BL antigens. In Burnet's terminology then, the "anti-BSA and anti-C57BL clones" must already be represented in thymus cells to the same extent that they later become among lymph node cells. In the present state of doubt about the range of specific action of a competent cell it is better to adhere to the simpler "inductive switch" hypothesis of thymus action, with its implication that each cell is multipotent, at this stage.

Study of the thymus would in this case be largely irrelevant to the first problem mentioned in the general introduction: that of the adaptability of the immune reponse. Neither would it throw light on the problem of immunological memory. There are many indications, however, that the thymus may be concerned with the development of auto-tolerance (or auto-paralysis). One of these is the association of thymic lesions with human auto-immune conditions (Miller, et al, 1962); and recently symptoms of auto-immunity have been induced in hamsters by direct injection of tissue homogenates into the thymus (Sherman, Adner and Dameshek, 1964). Another is the induction of paralysis specific to the thymus donor when a thmus allograft is implanted in a thymectomised mouse (Miller, 1962c). However, the thymus cannot be the only site where paralysis can be induced, since some antigens will induce complete paralysis in the adult within a time far too

short for complete turnover of competent cells (Dresser, 1962). But three considerations suggest that it could be a very suitable site for autoparalysis. One is the inhibition of immunisation which was mentioned in Part II. Another is the probability that the thymus acts as a bottle-neck through which all lymphoid cells have to pass during their maturation. Thirdly, since thymus cells have become determined on the lymphoid pathway they are more likely than are the bone marrow stem cells to bear the apparatus necessary for recognition of antigens. A further speculation, for which there is at present no evidence, is that the thymus might possess special means of processing tissue antigens into a paralytogenic form.

Arising more immediately from the work described in this thesis, the following points offer themselves to test:

1. The finishing school mechanism has so far been investigated with regard only to cells reactive to tissue antigens; but experiments similar to those described in Part II are now feasible with regard to antibody-producing cells. This could be done by transfer of thymus grafts between co-isogenic strains of mice differing only by a γ-globulin allotype, which would serve as a marker for determining the origin of the antibodies. It would be worth doing in view of the suggestions that allograft-type reactions may be served by a line of cells different from those involved in the production of circulating antibodies, both in birds (Warner, Szenberg and Burnet, 1962) and mammals (Sutherland, Archer and Good, 1964).

2. It should be possible to test the susceptibility to lasting paralysis by soluble antigens of lymphoid cells at 3 stages in their maturation before they become competent: (a) as bone marrow stem cells (b) in situ in the thymus, and (c) immediately after artificial release from the thymus. Again the use of allotype markers would help to identify the origin of the reacting cells, ~~from the paralysed ones~~.

3. In studies of lymphopoiesis by tritiated-thymidine labelling it has been shown that there are two distinct populations of lymphocytes: one short- and the other long-lived (Everett, Caffrey and Rieke, 1964). The significance of this is at present obscure, but it would be interesting, and perhaps illuminating, to look for changes in the proportions of these two types after thymectomy in adult life.

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# ABSTRACT OF THESIS

*Name of Candidate* Roger Broughton Taylor

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*Degree* Ph.D. *Date* October, 1964

*Title of Thesis* A Study of the Development and Maintainance of ~~Immunological~~ Responsiveness in Mice.

CBA mice have been thymectomised at various times from 1 day after birth up to adulthood, and tested subsequently for their responses to various antigens. The effect of neonatal thmectomy was to inhibit immune responsiveness generally. In particular, the response to bovine serum albumin (BSA) was strongly inhibited by thymectomy even as late as 7-14 days of age.

Immune responsiveness was restored to thymectomised mice by injections of lymph node or thymus cells, or by grafts of thymus tissue.

Amethod was devised to determine what proportion of the immunologically competent cells arose from these lymphoid tissue inocula, and what from the thymectomised hosts. The donor cells were distinguishable by their karyotype, being taken from the co-isogenic CBA/T6T6 strain of mice which bear a marker chromosome. In order to reveal the karyotype among competent cells these were caused to divide in the performance of a graft-versus-host reaction (GVH). The restored mice were killed and suspensions of their lymph node cells injected into (C57BL x CBA)$F_1$ recipients. In order to suppress mitosis among cells of the hybrid mice these were irradiated before receiving the transfer. The numbers of mitotic cells of each type were then scored in the hybrid spleens.

When the proportions of competent cells of donor and host origin, determined in this way, were taken together with the overall responsiveness (assessed by GVH spleen weight assay), it was concluded that:

(a) Thymus cells can gain an immune potency comparable to lymph node cells;

(b) Thymus grafts both donated competent cells to the thymectomised hosts, and brought about an increase in the number of competent host cells;

(c) Embryonic thymus grafts produced a smaller proportion of donor-type competent cells than did neonatal grafts;

(d) The thymus grafts became repopulated with host-type cells.

This evidence is discussed together with that of other workers in relation to 3 hypotheses of thymus action. It denies on the one hand that the thymus is the primary source of lymphoid stem cells, and on the other that its action is mediated pricipally by a circulating hormone. It favours instead the "finishing school" hypothesis: that the primary source of lymphoid stem cells lies outside the thymus, and that most of these cells pass throught the thymus in the course of their development.

In other experiments embryo liver cells were mixed with adult marrow cells and allowed to proliferate competitively in irradiated host mice. Cells from 12-day embryos (distinguished by the T6 marker) were as potent as adult marrow not only in the repopulation of marrow, but of thymus also. In a comparison of irradiated with normal marrow, made in the same way, irradiation depressed the proliferative capacity to the same extent (about 1%) whether this was assessed in marrow or

*Use other side if necessary.*

thymus. A proportion of cells bore a distinctive radiation-induced anomaly: these were present in both tissues. It is concluded that cells exist which combine lymphoid with haematogenous potentialities; and that since these cells were present in full numbers in embryo liver at xxx a stage before lymphocytes become evident in the thymus, the finishing school mechanism may well account for embryonic lymphopoiesis as well.

The responses of thymus cells were further investigated. It was found that the immune potency of these cells immediately after release from the thymus was only 3-10% of that of lymph node cells, in a number of test systems. But when they were allowed to reside in thymectomised host, mice thymus cells gained in potency within a week of transfer almost to equal lymph node cells. It is tentatively proposed that stem cells undergo an inductive change while in the thymus whereby they become determined towards a lymphoid form of differentiation, but that in order to become competent they require a further change: possibly release from a thymic inhibitory factor.

The effects of thymectomy in adult life were further studied, and the following results obtained:

1. Recovery of the immune response to BSA after irradiation was completely and lastingly suppressed.
2. Recover of responsiveness to BSA and BGG (bovine gamma globulin) after induction of paralysis by these antigens was partly inhibited.
3. In GVH assays at intervals up to 1 year after thymectomy the responsiveness remained normal for 25 weeks, then fell abruptly to 15% of normal. This fall was associated with a mild wasting condition.

It is concluded that the thymus is necessary in adult life for maintainance of responsiveness to its normal level. The results are further discussed in relation to theories of paralysis, and of lymphopoiesis. It is suggested:

(a) that recovery from paralysis depends mainly on the production of new competent cells rather than on the recovery of mature paralysed cells;

(b) to account for the prolonged maintainance and sudden decay of responsiveness after thymectomy in adult life, that a reserve of "precursor" cells exists, and that these can modulate to become competent cells until their own numbers are exhausted;

(c) to account for the maintainance of a basal level of responsiveness (15%) subsequent to this decay, and for the limited recovery from paralysis after thymectomy, that a low rate of lymphopoiesis can proceed in the absence of the thymus.