

ON THE HETEROGENEITY OF
MOUSE THYMOCYTES

Thesis submitted for the degree of
Doctor of Philosophy
in the Faculty of Science of the
University of London

by

Edward Victor Elliott, B.Sc., M.Sc.

Chester Beatty Research Institute,
Institute of Cancer Research,
Royal Cancer Hospital,
London, S.W.3.

January, 1975

ABSTRACT

Using an in vitro culture technique, mouse thymus graft cells have been cultured in the presence of phytohaemagglutinin (PHA). It has been shown that the cells responding by mitosis to PHA are generated within the thymus and are not part of the recirculating T-lymphocyte population. When grafts were examined at late times after grafting it was noted that a majority of the cells responding to PHA were of native thymus graft type; that is to say they had persisted in the thymus graft long after any mitotic evidence of their existence could be seen. These persistent cells were able to respond to other T-cell mitogens, and were relatively unaffected by either hydrocortisone or anti-lymphocyte serum treatment.

In allogeneic thymus grafts (AKR \rightarrow CBA), persistent PHA-responsive cells could be demonstrated, which were destroyed when incubated with anti- θ_{AKR} serum and complement. Thus indicating that the persistent cells are θ -positive.

When thymus graft cells were allowed to sediment on a 0.2 - 2% BSA gradient, it was seen that the PHA-responsive population sedimented faster than the bulk of thymus graft cells. Some separation could be obtained on this gradient between the "persistent" and "non-persistent" PHA-responsive cell populations.

Although not conclusive, many experiments tended to show that the stem cells responsible for the production of the "persistent" population were initially derived from outside the thymus, and not from thymic epithelial elements.

Experiments were performed showing that the "persistent" population did not leave the thymus in a number of situations, and when this population was injected into deprived recipients it did not localize in the spleen or lymph nodes but continued to circulate in the peripheral blood.

Due to the circulatory characteristics of this cell population, it could not be shown to be responding to the skin sensitizing agent oxazolone, or to foreign histocompatibility antigens in the graft versus host reaction (GvH). When however, it was allowed to react in vitro with foreign cells (MLR), it was over nine times less reactive to them than to PHA, when compared to the "non-persistent" population.

As yet no function has been demonstrated for the persistent PHA-responsive cell population, although it is postulated that it may play a role in the homeostasis of antigen reactive cells within the thymus.

ACKNOWLEDGEMENTS

Many people at the Chester Beatty helped and encouraged me during my second stay, 1970-1973. I would like especially to thank Dr E. Leuchars for the unselfish way she provided me with animals and time to carry out many of the various operational procedures, Miss V. Wallis for help in cytological analysis, Dr M. Doenhoff for showing me how to grow mouse lymphocytes, Mr C. Smith and his staff for supplying and caring for the experimental animals, Miss M. Butt for typing this thesis and Miss S. Gahr for drawing the graphs so well. I would like also to thank Mr K. Gomer, Miss C. Rees and Dr R. S. Kerbel for their help and advice.

Finally, to Dr A. J. S. Davies I extend my warm thanks for being my supervisor and encouraging me with his stimulating conversation throughout the course of this work.

"Science always was revolutionary and heterodox;
it is its very essence to be so; it ceases to be so only when
it is asleep."

G. Sarton (1927)

CONTENTS

	Page
Abstract	2
Acknowledgements	4
Introduction	7
Materials and Methods	26
Response of Mouse Thymocytes to PHA	45
Origin of PHA-responsive cells in the mouse thymus	53
Persistence of PHA-responsive cells in mouse thymus grafts	58
Response of native thymus graft cells to other mitogens	68
Effect of hydrocortisone and ALS on persistent PHA-responsive cells	73
Persistent PHA-responsive cells in allogeneic thymus grafts	81
Anti-theta(θ) sensitivity of persistent PHA-responsive cells from mouse thymus grafts	87
Sedimentation of mouse thymus graft cells	94
Bone marrow origin of persistent PHA-responsive cells	107
Recirculatory characteristics of persistent PHA-responsive cells	133
Immunological properties of persistent PHA-responsive cells	146
Concluding discussion	155
References	165

INTRODUCTION

During the last ten or so years it has been shown that the thymus has a significant role to play in immunity, both humoral and cellular. Lymphocytes can now be placed into two main categories, T-cells and B-cells. B-cells (bone marrow or bursal equivalent derived), in mammals are thought to originate in the adult from the bone marrow and to migrate to other peripheral lymphoid organs such as the blood, spleen and lymph nodes. These are the cells that produce the antibody in classical humoral immunity. T-cells (thymus-derived) on the other hand are produced in the thymus as a result of divisions of primitive stem cells that have previously migrated to the thymus from haematopoietic organs such as the bone marrow. T-cells do not produce these humoral antibodies which are detectable by the classical methods, but they can help B-cells in some unknown manner to produce antibody. It has also been shown that some T-cells have the potential to kill other cells, and they are also probably the main type of effector cells seen in graft rejection, graft-versus-host reactions and cytotoxic reactions against tumour cells.

We seem to know quite a lot about T-cells after they have left the thymus (see Greaves, Owen and Raff, 1973), but relatively

little with regards to their production in the thymus. This introduction will be concerned with the production and characteristics of cells of the T-cell series within the thymus, and will deal mainly with developments in the field that have taken place since Miller and Osoba's review in 1967.

Origin of the Thymus and Thymic Lymphocytes

The thymus anlage arises from epithelial regions of ventral portions of the third and fourth branchial pouches (Venzke, 1952). It is initially epithelial but later becomes surrounded by mesenchyme (Auerbach, 1960). It can be dissected out of the mouse embryo after only eleven days of gestation before it becomes lymphoidal (Owen and Ritter, 1969). In a series of elegant experiments, Auerbach (1961, 1963, 1964) cultured thymic rudiments from 12 day old mouse embryos both in vivo and in vitro. Mesenchyme was separated from epithelial tissue by trypsinization and the various residues grafted into the anterior chamber of the eye or cultured in vitro. Auerbach suggested that the mesenchyme was responsible for the initial induction stimulus for morphogenesis but that the epithelium was the source of the lymphocytes. The evidence that lymphocytes develop from the epithelium does not however exclude the possibility that lymphoid

precursor stem cells had already entered the thymic epithelial rudiment. In part, to answer this question, Moore and Owen (1967a) parabiosed male to female chicks in ovo at various days after fertilization. Male cells can be distinguished from female cells by the presence of two sex chromosomes (females have only one). They found that in chicks parabiosed 4 days after fertilization, up to 50% of the cells dividing in the thymus were from the other partner. Thus from these experiments it would seem that stem cells capable of dividing in the thymus were blood borne and not as Auerbach suggested, derived from the thymic epithelium. Further experiments by Moore and Owen (1967b) showed that cells with a prominent nucleolus and basophilic cytoplasm could be found in mouse and chick embryo thymuses. The earlier the embryo thymus was examined, the fewer of these cells could be seen. When these rudiments were grafted onto the chick chorioallantoic membrane and examined for the presence of the chromosome marker four days later, they showed that when few or no basophilic cells were present in the rudiment, the thymus was repopulated by host cells, whereas older grafts grew from their own cells at least for a time. This evidence suggested that the basophilic cells were stem cells which had migrated to the thymus in the embryo. Further experiments by Owen and Ritter (1969), who grew mouse and chick embryo thymuses in Millipore

chambers placed on the chick chorioallantoic membrane, showed that grafts without basophilic cells failed to grow, whereas those with basophilic cells grew and contained lymphocytes. This evidence again suggested that the basophilic cells were stem cells which had migrated to the thymus.

Overwhelming evidence has also been obtained to suggest that neonatal thymus grafts and regenerating adult thymuses are repopulated eventually from cells which originate from outside the thymus. This has been shown using chromosome markers (Dukor et al., 1965; Leuchars et al., 1967; Micklem et al., 1966) and the θ antigen (Owen and Raff, 1970).

It will be seen that in contrast to the older opinions of Auerbach and Sainte-Marie and Leblond (1964a & b, 1965), most workers seem now to concur with the idea that all thymic lymphocytes develop from stem cells which enter the thymus via the blood.

The Thymic Epithelium

It has previously been shown that the thymus develops after intimate contact between epithelium and mesenchyme, and that lymphocytes develop from stem cells that enter the thymus from the blood stream. It thus appears that the thymus itself is in a sense a chimaeric organ with epithelial elements and lymphoid

elements derived from two different sources. Miller and Osoba (1967) have suggested that thymus grafts eventually come to consist of epithelial elements of thymus graft donor-type while the lymphoid compartment is replaced by bone marrow-derived cells. There is little information about the durability of the thymic epithelium itself, although Hayes (1969) has suggested that the epithelial cells in the thymus persist for much longer periods than the majority of thymic lymphocytes. It has been shown that some thymic epithelial cells at least can incorporate tritiated thymidine during early postnatal life, and that the label persists in these cells for at least 42 days, again suggesting that the thymic epithelial-reticular framework is long-lived (Parrott and de Sousa, 1967).

The only function attributed to the thymus epithelium is that of "thymosin" or "thymus-humoral factor" production (Mandi and Glant, 1973; Vettors and Macadam, 1973). Whether this factor, which is undergoing intense research at the moment, has any relevance in the maturation of thymic lymphocytes within the thymus, has yet to be elucidated.

Thymic Stem Cells

We know very little concerning the identity of the thymic

stem cell. It has been suggested previously that it has a prominent nucleolus and strongly basophilic cytoplasm. There is evidence to suggest that the stem cell responsible for the production of spleen colony forming units (CFU) in lethally irradiated mice can under certain conditions give rise to lymphocytes (Wu et al., 1968). Thus it seems that the stem cell responsible for the development of the thymus is originally multipotential, but that when it becomes extravascularized in the thymus it will divide and differentiate into thymic lymphocytes. Metcalf and Moore (1971) have found that cells from 12-day mouse embryo thymuses can give rise to CFU's, suggesting that thymic stem cells only become committed after entering the thymus.

It would appear that stem cells enter the thymus anlage early in embryogenesis (7 days in the chick - Owen and Ritter, 1969). During this period the yolk sac is the main haematopoietic organ and is therefore their most likely source. In the mouse embryo large basophilic cells similar in appearance to those found in the developing chick thymus, can be seen at 11 days of gestation. It seems likely that these stem cells are derived from either the yolk sac and/or the foetal liver. Indeed Tyan (1968) showed a sequential appearance of stem cells in the yolk sac and foetal liver, and that at 28 days after birth they could be found only in the bone

marrow of the mouse.

It seems likely that thymic stem cells are not immortal, but continue to enter the thymus during life. This has been shown using chromosome markers, in adult parabiosed mice (Harris et al., 1964) and in thymus grafts (Dukor et al., 1965; Leuchars et al., 1967). Indeed repopulation studies of irradiated thymuses show that such organs are repopulated eventually by cells of bone marrow origin (Micklem et al., 1966).

We do not know how many stem cells are required to initiate the growth of the embryonic thymus, but we do know that in the adult the numbers are small since even 30 weeks after parabiosis the level of thymic chimaerism was only 15-18% (Harris et al., 1964). More recently, experiments looking at the regeneration of irradiated thymuses by different numbers of bone marrow cells, have suggested that these thymuses can be initially regenerated by as few as one stem cell (Wallis et al., 1974).

Once the stem cell has reached the thymus, nothing is known concerning the events which allow it to develop into a lymphoid cell population. Indeed this differentiation pathway still remains a complete mystery although it seems likely that there is some interaction between cells of the thymic framework and the stem cell. What is known about these stem cells however is that initially

in the thymus they do not possess certain of the characteristic thymic antigens, but that shortly after, their progeny become positive for such antigens. Thus Owen and Raff (1970) showed that the large basophilic cells found within 14 day old mouse embryo thymuses were negative for both the θ and TL antigens. If these thymus rudiments were cultured for 4 days in vitro, the lymphocytes resulting from such a procedure were positive for both the θ and TL antigens. Further in vitro studies using embryo mouse thymuses by Ritter (1972) have shown that the differentiation step from stem cell to thymocyte is accompanied by an increase in sensitivity to the effects of irradiation and corticosteroids. It has also been shown that the cell in adult bone marrow which is capable of repopulating the adult irradiated thymus is also negative for certain thymic antigens (Boyse and Old, 1969).

Thymic Lymphopoiesis

From the previous section it would seem that the thymic stem cell enters the thymus and proceeds to divide rapidly due to some signal from the thymic framework. The end result of these divisions is the population of the thymus cortex. Later on in thymic development the thymus medulla is laid down. At first

glance, the main difference between the (outer) cortex and the (inner) medulla appears to be the cellularity. The cortex contains masses of closely packed lymphocytes, especially in its outer region, whereas the medulla appears much more sparsely populated by lymphocytes. Cortical lymphocytes would appear to comprise approximately 85-90% of all thymic lymphocytes in the mouse.

Morphological examination of thymic lymphocytes has shown that the mitotic index of the thymus is much higher than in other lymphoid organs (Andraesen and Christensen, 1949). There is at least one area of intense mitotic activity in the thymus, and this is in the area immediately beneath the thymic capsule. Autoradiographic studies utilizing pulse labels of tritiated thymidine have indicated that the label initially accumulates in the area immediately beneath the thymic capsule. Within a short time the labelled cells migrate inwards towards the medulla where they eventually end up after a few days before leaving the thymus (Everett & Tyler, 1967; Metcalf, 1966; Borum, 1965, 1968, 1973). A recent report suggests that there might be two areas of mitotic activity in the guinea pig thymus, the first being beneath the thymic capsule and the second at the corticomedullary junction (Poste and Olson, 1973). Even during age involution, the thymus cortex would still seem to have the highest basal mitotic rate of all lymphoid

organs (Andraesen and Christensen, 1949).

One of the best methods for studying the characteristics of the medullary population is to inject animals with corticosteroids. Cortical lymphocytes are very sensitive to corticosteroids and appear to undergo a rapid death and elimination, whereas medullary lymphocytes are much more resistant to the cytolytic effects of corticosteroids (Ishidate and Metcalf, 1963; Dougherty et al., 1964). This treatment enables one to study a relatively pure population of medullary lymphocytes, but as yet no agent has been found which will destroy the medulla but leave the cortex unaffected. Other agents or treatments have been shown to affect the thymus; thus involution of the thymus occurs after hypophysectomy (Lunden, 1958) while there is an increase in thymus weight following adrenalectomy (Ishidate and Metcalf, 1963).

It appears that the growth of the thymus is not controlled by the same mechanisms which control the growth of say the spleen. Thus it is of interest to note that removal of one thymus lobe from the mouse does not cause an increase in the weight of the remaining thymic tissue (Metcalf, 1964). Also it would appear that antigen does not seem to affect the thymus, since no differences in thymic tissue could be seen between germ-free and normal animals (Gordon, 1959).

Thymic Antigens

The antigenicity of thymocytes has been extensively reviewed recently by Schlesinger (1972), and this section will deal briefly with some of the antigens present on mouse thymocytes.

(1) H₂ Antigens

The majority of mouse thymocytes have much less H₂-antigen on their surface than do lymph node or splenic lymphocytes (Winn, 1960, 1962). Winn showed that certain dilutions of anti H₂ antisera, in the presence of guinea pig complement, were able to kill 100% of lymph node lymphocytes but only between 8-15% of thymic lymphocytes. Thus it is possible that the small percentage of thymocytes killed by those concentrations of antisera Winn used, are situated in the thymus medulla and that medullary thymocytes have more H₂ antigen on their surface than do cortical thymocytes.

(2) Ly Antigens

Ly antigens are present on mouse thymus, lymph node, spleen and bone marrow cells. Boyse and his coworkers (1968) by absorption studies showed that lymph node, spleen and bone marrow cells had about 65%, 50% and 5% respectively of the concentration of Ly antigen found on thymus cells, but no comparison was made between cortical and medullary thymocytes.

(3) TL Antigen

The TL antigen was first discovered by Boyse and his

co-workers (1963). They later indicated that mouse strains could be divided into two groups; TL-positive and TL-negative strains (Boyse et al., 1965). In TL-positive strains the antigen is found only on thymus cells but not on T-cells in the periphery. In TL-negative strains the antigen is found neither on thymocytes nor on peripheral T-cells. In both groups however the TL-antigen is present on certain leukaemias. There is evidence that in the thymus the cortical lymphocytes possess the TL-antigen whereas the medullary lymphocytes do not (Leckband and Boyse, 1971; Leckband, 1970; Raff, 1971a).

(4) θ Antigen

Reif and Allen (1963, 1964) described two alleles of the θ antigen in mice: θ -AKR and θ_{C_3H} . The highest concentrations of θ -antigen in the mouse are found in the thymus and the brain, and in the thymus both cortical and medullary lymphocytes possess surface θ antigen. It has been shown that stem cells capable of repopulating the thymus have no surface θ antigen but that soon after cortical mitoses begin, θ is expressed in high concentrations on the cortical lymphocyte surface (Owen and Raff, 1970). At some stage it is believed that cells traverse the corticomedullary junction and are found in the medulla (Borum, 1968). At some stage during this migration a large amount of θ antigen appears to be lost from

the cell surface ($\theta^{+++} \rightarrow \theta^+$), since it has been shown that higher concentrations of anti- θ serum and complement are needed to kill corticosteroid-resistant thymocytes than are needed to destroy the majority of normal thymocytes (Raff, 1971a). It thus appears that medullary thymocytes, like peripheral T-cells, have much less θ antigen on their surface than do cortical lymphocytes.

With most of the antigens so far described there appear to be quite large differences between cortical and medullary thymocytes. Whether all these changes occur at the same time and location within the thymus, or whether cells in the medulla derive from different stem cells without passing through the cortex is as yet unknown.

There are a multitude of other antigens demonstrated on thymocytes of the mouse and other species which will not be described here, and even during the last few months an antisera prepared by injecting rabbits with certain plasma cell tumours, have, after appropriate adsorption, the capability of killing, along with complement, 50% of mouse thymocytes (Yutoku et al., 1974). An even more inexplicable report suggests that the sera of schizophrenic patients, with complement, are cytotoxic for mouse thymocytes, whereas the sera of normal subjects are not cytotoxic (Luria and Domashneva, 1974)!

Medullary Thymocytes

Many investigators in the early 1960's noted that it required large numbers of normal thymocytes to restore immunological competence to T-cell deprived mice, in contrast to the much smaller numbers of lymph node or spleen cells needed (reviewed by Miller and Osoba, 1967). It was suggested that this immunological restoration was brought about by the incorporation of some thymus cells into the T-cell pool of the recipient mice, and because large numbers of thymus cells were needed for good restoration ($> 100 \times 10^6$ cells), that the cells capable of restoring T-cell deficiencies were a minority of the thymocyte population. Since that time it has become much more evident that the thymus did contain a minority population of immunologically competent cells.

With the use of corticosteroids (vide supra), it has been possible to deplete the thymus almost entirely of the cortical cell population. When the residual medullary cells were used as a source of immunologically competent cells, it was seen that far fewer cells were needed to restore immunological competence to deprived mice, than normal untreated thymocytes (Andersson and Blomgren, 1970).

Corticosteroid-resistant thymocytes (CRT) are used synonymously with medullary thymocytes in the rest of this thesis.

These cells can respond to PHA (Colley et al., 1970; Blomgren

and Svedmyr, 1971; Elliott et al., 1971) and other mitogens (reviewed by Greaves and Janossy, 1972). They can restore immunological function to thymus deprived animals (Transplantation Reviews, 1, 1969) and be activated by certain antigenic stimuli; for example by passage into irradiated animals along with antigen they can specifically cooperate with B-cells in the production of antibody (Andersson and Blomgren, 1970); they can initiate graft-versus-host reactions (Blomgren and Andersson, 1969), mixed lymphocyte reactions (Knight and Thorbecke, 1971), and destroy tumour cells (Cerottini et al., 1970).

The medullary cell population would appear to make up less than 10% of the total thymocyte population and consist of cells which are not in division. It is comprised of cells which are larger but less dense than the cortical population (Colley et al., 1970; Konda et al., 1972; Shortman et al., 1972a,b).

When separated electrophoretically, thymocytes can be placed into populations of high and low electrophoretic mobilities (Nordling et al., 1972; Zeiller and Dolan, 1972; Wiig, 1973). The medullary population appears to have the high electrophoretic mobility associated with peripheral T-cells (Wiig, 1973) whereas the cortical thymocyte population has a slow electrophoretic mobility similar to the peripheral B-cell population. Indeed it has been suggested recently, on the basis of electrophoretic mobility, that thymocytes

can be separated into five distinct subpopulations (Zeiller et al., 1974).

A rapidly growing number of experiments have suggested that there are cells in the thymus which can suppress immune responses when transferred to suitable recipient animals (Droege, 1971; Gershon et al., 1972; Ha and Waksman, 1973). These cells are termed "suppressor T-cells" (reviewed by Gershon, 1973). Furthermore, Ha, Waksman and Treffers (1974) have suggested that these "suppressor" cells in the thymus reside in a subpopulation of low density, making up less than 10% of the total thymocytes, and are partially resistant to hydrocortisone.

It would appear from all these experiments that the medulla contains cells which have "positive" and "negative" immunological functions. Unless these functions are carried out by the same cell population, then it would appear that the medullary cell population is heterogeneous.

Emigration of Cells from the Thymus

There is now overwhelming evidence that some lymphocytes at least, leave the thymus (Miller, 1962; Weissman, 1967; Davies, 1969) and become incorporated into the peripheral T-cell population where they are known as T-lymphocytes (Roitt et al., 1969).

It has been suggested by Metcalf (1966) that most of the cells

produced in the thymus eventually die there, but there is very little evidence to support this hypothesis and it now seems that a majority of thymocytes leaving the thymus die elsewhere, since it cannot be shown that they are incorporated into the T-cell pool. Evidence for this notion was provided by Ernström and his coworkers (Ernström et al., 1965; Ernström and Larsson, 1967; Ernström, 1969). They showed by measuring the concentration of lymphocytes in the afferent and efferent blood vessels of the guinea pig thymus, that there were many more lymphocytes present in the thymic vein than in the carotid artery. Also following tritiated thymidine labelling of guinea pigs they showed that the number of labelled cells was much higher in the thymic vein than in the carotid artery, and from these measurements they estimated that the number of cells leaving the thymus was sufficient to replace the peripheral lymphocyte population once every six hours. The one drawback to these experiments is that the surgical procedures undertaken, could themselves have caused the effects seen. Ignoring this proviso, and knowing that the peripheral T-cell population is long lived and added to slowly in adult life (see Greaves, Owen and Raff, 1973), then it would seem that a vast majority of the cells leaving the thymus must have a very short life-span.

Further evidence for the emigration of cells from the thymus

to the periphery has been provided by the use of chromosomally marked thymus grafts in T-cell deprived hosts. Thus soon after grafting, cells of known thymus genotype can, under appropriate condition, be found in peripheral lymphoid organs (Miller et al., 1966; Davies, 1969). Furthermore the gradual dilution of cells in the periphery of known thymus origin which occurs following grafting, can be halted by the removal of the thymus graft (Doenhoff et al., 1970a). Similar experiments, this time using CBA thymus grafts (θ_{C3H}) to repopulate deprived AKR mice (θ_{AKR}), have shown that cells of known thymus graft origin can leave the thymus and be found, with the use of the appropriate anti- θ serum, in peripheral lymphoid organs (Owen and Raff, 1970).

Studies which show that neonatally thymectomized mice; adult thymectomized, irradiated, bone-marrow injected mice, and congenically athymic nude mice all lack the capacity to give good responses of the T-cell mediated type, is further indirect evidence that the thymus is important in the liberation of immunologically competent T-lymphocytes. Furthermore, the same inference can be made from the embryological evidence that the thymus is the first organ in the body to possess lymphocytes, followed shortly after by other lymphoid organs (reviewed by Miller and Davies, 1964). Also Auerbach's experiments with embryonic thymus and spleen cultures

indicate that the embryonic spleen does not become lymphoid until after the presence of thymic lymphocytes, and that the lymphocytes found in the spleen were derived from the thymus (Auerbach, 1963).

In the preceding discussion it will be noted that no mention was made of Hassell's corpuscles, PAS positive cells or macrophages, all of which can be found in the thymus. This omission was due to the fact that these topics have been extensively reviewed by Miller and Osoba (1967), and little new work has come to light since that time.

Many other facts concerning thymocytes have been omitted from this review; for instance the fact that certain cells in the thymus can bind antigen (Modabber et al., 1970; Bach, 1971), while others appear to possess the receptor for the Fc portion of IgG (Yoshida and Andersson, 1972) is of interest.

There seems to be an awesome number of facts concerning the thymus which as yet are inadequately correlated. It is hoped that workers in the future will come to understand more about the differentiation pathway steps that occur in the organ, so that another golden age of "thymology" may dawn (see Miller, 1967).

MATERIALS AND METHODS

MICE

Three strains of CBA mice which are chromosomally distinct but nevertheless syngeneic, were used interchangeably as hosts in most of the experiments reported. These three strains are CBA/Lac, CBA/H.T6T6 and (CBA/Lac x CBA/H.T6T6) F_1 hybrids, the cells of which can be distinguished at metaphase by the presence of none, two or one minute marker chromosomes respectively (Figs. 1a, 1b and 1c).

In some experiments AKR, BALB/c, and (BALB/c x CBA/Lac) F_1 mice were used as either thymus graft donors or as a source of stimulator cells in the mixed lymphocyte reaction.

THYMECTOMY

Mice were thymectomized at approximately 6 weeks of age according to the method of Miller (1960). This involved making a one inch mid-ventral incision of the skin with scissors in the cervical and upper sternal region. The salivary glands were parted and a portion of the sternum was removed. The thin membrane covering the thymus was removed and the two thymus lobes were removed using a suction pump. Gentle pressure was put on the thorax at this stage since failure to do

Figs. 1a, 1b, 1c.

Colcemid-arrested metaphase plates of
PHA-stimulated CBA mouse lymphocytes.

Fig. 1a CBA/Lac

Fig. 1b CBA/H.T6T6

Fig. 1c (CBA/Lac x CBA/H.T6T6) F_1 hybrid

Note the minute T6 chromosomes.

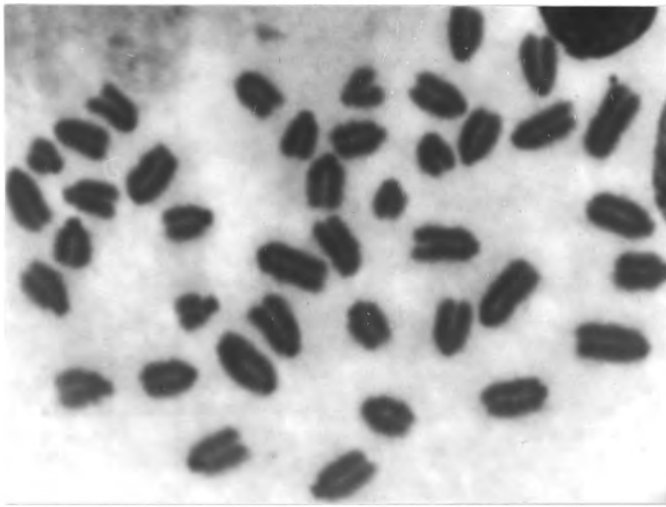


fig. 1a

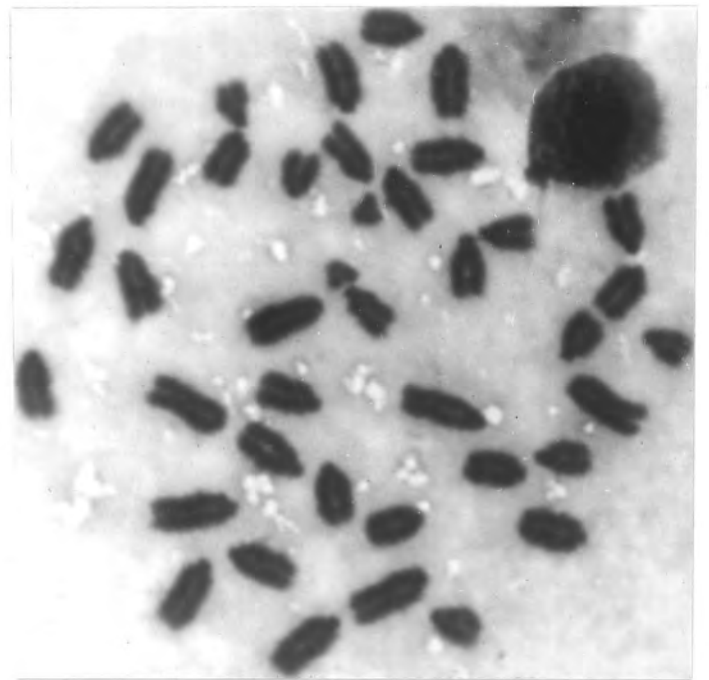


fig. 1b

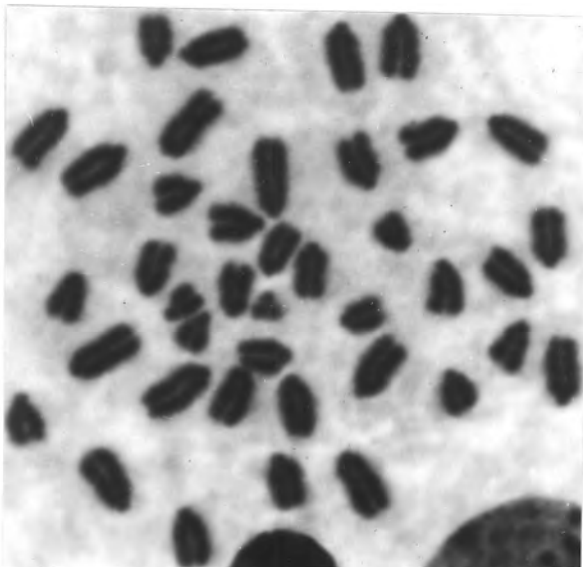


fig. 1c

so can easily result in a pneumothorax. Finally the two flaps of skin were drawn together and joined with steel clips and the animal allowed to regain consciousness. Thymectomy was carried out under ether anaesthesia, and post operative death was negligible.

IRRADIATION

Mice were generally irradiated 7-10 days following adult thymectomy. 850r was delivered to the whole body from a 220 kV Westinghouse X-ray machine at a focal distance of 100 cms and a dose rate of 60r per minute. For irradiation, mice were housed in ventilated perspex boxes.

In one experiment neonatal thymuses were irradiated in vitro at a focal distance of 20 cm from the X-ray source, for 40 seconds at 1000r/minute.

BONE MARROW THERAPY

Within three hours of irradiation, syngeneic bone marrow was administered via the tail vein. The contents of two femurs were dispersed in medium 199 through a 23 gauge needle and used to reconstitute five recipient mice. From previous studies in this laboratory, each recipient animal received approximately 5×10^6 nucleated cells.

In one experiment, irradiated mice received numbers of

nucleated bone marrow cells ranging from 1×10^7 down to 1×10^5 intravenously.

THYMUS GRAFTING

Thymuses were removed from 1-3 day old mice aseptically. The thymus lobes were separated and implanted under the kidney capsule (or subcutaneously) in the host mice (Davies et al., 1966). The sex of the thymus graft donor was not noted.

a) Numbers of thymus grafts

The subcutaneous route for thymus grafting was used when large numbers of thymus grafts were needed at later times, and up to 20 separate lobes have been grafted in this way. There were no observable differences in the results obtained with subcapular or subcutaneous thymus grafts.

b) Types of thymus grafts

Neonatal CBA (Lac, T6T6 or (Lac x T6T6)F₁) thymuses can be successfully grafted between all three strains without any signs of rejection. CBA or AKR thymuses were grafted into deprived CBA mice usually within four hours of irradiation and bone marrow therapy, whereas BALB/c thymuses were grafted 10 days after deprivation.

TREATMENT OF NEONATAL THYMUSES BEFORE GRAFTING

In some experiments the neonatal thymuses were treated in certain ways before grafting.

a) No treatment

Neonatal thymuses were removed and kept in medium 199 on ice before grafting.

b) Irradiation

As mentioned previously, some neonatal thymuses were subjected to 667r irradiation in vitro (in 199).

c) Cut ends and squashing

In one experiment individual thymus lobes were placed on a glass slide. The ends of the thymus were cut off with a scalpel blade and the cells squashed from the thymus by placing on top of the thymus another glass slide and exerting gentle pressure. Almost all cells were released from the thymus, and the remnant was grafted under the kidney capsule of a recipient mouse. The control in this experiment were thymuses which just had their ends cut off but were not squashed.

d) Thymus grafts in millipore chambers

In another experiment neonatal thymuses were placed in cell impermeable millipore chambers (0.2 micron porosity) sealed with acetone. These chambers were placed into the

peritoneal cavities of recipient mice. After 5 or 11 days the mice were killed and the chambers removed. The thymus graft remnants were removed and grafted under the kidney capsules of further recipient mice.

THYMOCYTES FOR INJECTION

In some experiments normal or hydrocortisone-resistant thymus graft cells were injected intravenously into deprived mice (thymectomized, irradiated and bone marrow injected). These thymuses were teased through stainless steel sieves and the cells washed at least 4 times in medium 199 before being injected.

PREPARATION OF CELLS FOR CULTURE

a) Peripheral blood lymphocytes

The method used for the preparation of relatively pure mouse peripheral blood lymphocytes for culture with mitogens was essentially that of Festenstein (1968) and Doenhoff et al. (1970a). Mice were killed by asphyxiation with CO₂ (from 'dry ice'). After swabbing down the mice with cetrimide, the thorax was opened, and 0.25 ml of phosphate buffered saline containing 3.5% trisodium citrate (isotonic) was injected into the left ventricle of the heart. After a few seconds the ventricles were cut with scissors and the resulting pool of blood placed into a

2.5 ml sterile plastic tube (Staynes Laboratories Ltd.) via a sterile Pasteur pipette. To each tube of blood was added two (2-3 mm diameter) glass beads and 0.15 mls of 10% calcium gluconate. The tubes were shaken for 4-5 minutes to defibrinate the blood. To this defibrinated blood was added 1 ml Plasma gel (Roger Bellon, France) and the mixture was aspirated. After standing at room temperature for approximately 30 minutes, the erythrocytes were seen to have sedimented and the supernatant was transferred to a clean tube and centrifuged at 600g for 10 minutes. The pellet was resuspended in culture medium (Festenstein, 1968).

This method allowed two replicate cultures to be prepared from the same mouse, or, by scaling up all procedures to make pools of peripheral blood lymphocytes from several animals. In most experiments to be described, a pool of relatively pure (> 94%) mouse peripheral blood lymphocytes (Doenhoff et al., 1970a) was counted in a Coulter Counter, and after dilution to 2×10^6 nucleated cells per ml, mixed in certain proportions with cells from thymus grafts, and the cell mixtures were cultured with PHA.

b) Thymic lymphocytes

Thymuses or thymus grafts were removed from animals and teased individually through stainless steel sieves into medium

199. After washing the cell suspension at least two times in medium 199, the cells were made up in culture medium (Festenstein, 1968) and counted. All cell suspensions were diluted to 2×10^6 nucleated cells per ml and in most experiments mixed in certain proportions with peripheral blood lymphocytes and cultured with PHA.

c) Lymph node and splenic lymphocytes

In some experiments, splenic and/or lymph node lymphocytes were prepared by teasing spleens and/or lymph nodes individually through a stainless steel sieve into medium 199. After washing the cell suspensions at least twice in medium 199, the cells were made up in culture medium (Festenstein, 1968) and counted. For culturing, all cell suspensions were diluted to 2×10^6 nucleated cells per ml.

MITOGENS

a) Phytohaemagglutinin (PHA)

Purified PHA (Burroughs Wellcome, dried) was used to stimulate proliferation in mouse lymphocyte cultures. 0.05 ml of PHA diluted 1/8 in phosphate buffered saline (PBS) was added to 1 ml cultures of mouse lymphocytes (2×10^6 cells). This gave a final dilution of 1/160 (approximately 2.5 μg /culture) which has been

shown to give maximal incorporation of tritiated thymidine between 2-3 days of culture (Doenhoff, 1970).

b) Concanavalin A (Con A)

Con A was obtained from Pharmacia, Sweden, and dissolved in PBS. Model experiments showed that maximal tritiated thymidine incorporation between 2-3 days of culture occurred at a concentration of 5 µg (in 0.05 ml) per 1 ml culture of 2×10^6 hydrocortisone-resistant thymocytes.

c) Pokeweed mitogen (PWM)

A solution of PWM dissolved in PBS was used at a concentration of 50 µg (0.05 ml) per 1 ml culture containing 2×10^6 lymphocytes.- This mitogen was purified from the plant stems of Phytolacca americana using the method described by Borjeson et al. (1966).

d) Lipopolysaccharide (LPS)

E. coli LPS was obtained from Difco Laboratories (0.55: B5) and diluted in PBS. LPS does not stimulate normal thymocytes, hydrocortisone-resistant thymocytes (Greaves and Janossy, 1972) or peripheral blood lymphocytes (Doenhoff, unpublished observations), but does stimulate spleen cells. The concentration of LPS which caused maximum uptake of tritiated thymidine between 2 and 3 days

of culture was 50 µg per 1 ml culture of spleen cells (2×10^6 cells per ml), and this concentration was used in the experiments described in a later section.

e) Staphylococcal enterotoxin B (SEB)

5 mg of SEB was kindly provided by Dr W. Adler. It was dissolved in 5 ml of PBS, and model experiments suggested that maximum tritiated thymidine uptake by hydrocortisone-resistant thymocytes (between 2 and 3 days of culture) occurred at a concentration of 50 µg per 1 ml culture (2×10^6 cells).

CELL CULTURES

a) Constituents of medium

Sterile glass distilled water

Eagles Minimal Essential Medium (MEM) without antibiotics or

sodium bicarbonate (Burroughs Wellcome, 10x concentrated)

Foetal calf serum (Difco or Flow)

Sodium bicarbonate (Burroughs Wellcome)

Antibiotics - sodium cloxacillin (Beecham)

- ampicillin (Beecham)

Mitogens (various)

b) Preparation of medium

Sterile stock solutions were prepared. 7.5 ml Eagles MEM was mixed with 7.5 ml heat inactivated foetal calf serum ($56^{\circ}/30'$)

and added to 64.5 ml sterile distilled water. To this mixture was added 2.55 ml sodium bicarbonate and 1.05 ml antibiotic solution (250 mg of each antibiotic was dissolved in 20 ml sterile distilled water). Cells were resuspended in this medium at a final concentration of 2×10^6 cells per ml, and 1 ml was cultured in 2.5 ml flat-bottomed polystyrene tubes (Staynes Labs.) with the appropriate mitogen added.

c) Conditions of culture

The caps of the culture tubes were punctured with a needle and the tubes incubated in a desiccator in an atmosphere of 8% CO₂ and 92% N₂ at 33°C for 3 days in most experiments.

CYTOLOGICAL ANALYSIS

Cell cultures received Colcemid (Ciba) at a concentration of 0.1 µg per ml, either 16 hours or 5 hours before harvesting. At harvesting, the medium was removed and replaced with 1% hypotonic sodium citrate solution and the preparation of metaphase plates for karyotype analysis was carried out according to the method of Ford (1966).

In some cases where cytological analysis of lymphoid organs was essential, 4 µg of Colcemid per gm body weight was injected intraperitoneally, and tissues were removed $1\frac{1}{4}$ hours later

for cytological analysis. In most cases at least 50 metaphase plates were scored per culture or organ. Where possible, when an irrelevant cell population needed to be counted, 50 metaphase plates of the relevant cell population were counted.

TRITIATED THYMIDINE (H_3T) INCORPORATION

Some cell cultures stimulated with PHA were assessed for their ability to incorporate H_3T . Approximately $5\mu Ci$ H_3T were added to each culture after about 54 hours incubation. The cultures were incubated at $33^{\circ}C$ for a further 16 hours overnight and the amount of H_3T incorporated into 0.1 ml of cell suspension (1 ml cultures) was assessed. Briefly, 0.1 ml of cell-free supernatant from each culture was spread on a filter disc (Whatman 3MM, 2.3 cm diameter) to yield a supernatant control. The remaining cells and medium were resuspended and 0.1 ml aliquots were spread on two filter discs to give duplicate samples. The discs were dried at room temperature, after which they were fixed in 10% trichloroacetic acid (20 min), washed in 3% perchloric acid (20 min) and rinsed twice in methanol and once in ether. After drying, the discs were placed in counting vials with 10 ml scintillation fluid and assayed for radioactivity in a liquid scintillation counter (Tricarb Model 3320). The counts per minute (c.p.m) of the supernatant

control was subtracted from the mean of the two cell sample discs. The results are expressed as c.p.m. per 0.1 ml of cell culture.

MIXED LYMPHOCYTE REACTION (MLR)

In some experiments hydrocortisone-resistant cells from thymus grafts were set up in mixed lymphocyte cultures with allogeneic cells. In these experiments 4×10^6 hydrocortisone-resistant cells (CBA) were cultured with either 4×10^6 BALB/c spleen cells, 4×10^6 Mitomycin C blocked ($25 \mu\text{g MC}/30'/37^\circ\text{C}$) BALB/c spleen cells, or 4×10^6 CBA x BALB/c spleen cells, in 4 ml of medium (previously described) in 5 ml flat-bottomed polystyrene tubes (Sterilin) for 5 days at 33°C . In model experiments, 5 days of culture was shown to allow maximum tritiated thymidine incorporation during the last 16 hours of culture when compared to other times tested. After 5 days the cultures were assessed for mitotic figures.

GRAFT versus HOST REACTION (GvH)

The ability of CBA hydrocortisone-resistant thymus graft cells to cause a GvH reaction in (CBA x BALB/c) F_1 mice was assessed. Briefly, 20×10^6 CBA hydrocortisone-resistant thymus graft cells were injected intravenously into lethally irradiated (850r) (CBA x BALB/c) F_1 animals. Four days later colcemid was injected

and the spleen removed $1\frac{1}{4}$ hours later for cytological analysis. The 4-day GvH response gave the maximum number of mitotic figures in model time course experiments.

HYDROCORTISONE SODIUM SUCCINATE (HSS)

Hydrocortisone sodium succinate (HSS) was obtained from Organon Laboratories. It was dissolved in saline and each mouse received 10 mg intraperitoneally. This dose resulted in the reduction of thymus weight to less than 10% of control values by 3 days, and these thymocytes were designated "hydrocortisone-resistant thymocytes".

ANTI-LYMPHOCYTE SERUM (ALS)

Rabbit anti-mouse ALS was prepared according to the method of Levey and Medawar (1966). 1×10^9 washed mouse thymocytes were injected intravenously on two occasions (14 days apart) into a rabbit. The rabbit was exsanguinated 7 days after the second injection, and the serum was heated to 56°C for 30 minutes and stored in 10 ml aliquots at -20°C . 0.5 ml of this serum was injected subcutaneously into mice on two occasions (2 days apart), and in a model experiment the PHA response of peripheral blood lymphocytes 3 days after the second injection showed a fall to 3.7% of control values. Thus, this injection

regimen was used in the experiment to be described in a later section.

OXAZOLONE

In some experiments the skin sensitizing agent oxazolone (2 phenyl-4-ethoxymethylene-5-oxazolone) was used. Mice were painted with a 3% solution of oxazolone in absolute alcohol. This solution was applied using a camel haired brush, the dose being equally divided between the two fore feet and two shaved sites high up on the back. At three days the draining internal and external axillary lymph nodes were removed for cytological analysis after in vivo colcemid treatment.

PREPARATION OF ANTI- θ _{C3H} and ANTI- θ _{AKR} SERA

These sera were prepared according to the method of Rief and Allen (1964).

a) Anti- θ _{C3H}

AKR mice were injected intraperitoneally with 1×10^7 CBA/Lac thymocytes on six occasions at weekly intervals. Ten days after the final injection the mice were exsanguinated and the serum was heated at 56°C for 30 min and stored at -20°C .

b) Anti- θ _{AKR}

CBA/Lac mice were injected intraperitoneally with 1×10^7 AKR thymocytes (from young mice) on six occasions at weekly intervals. Ten days after the final injection the mice were exsanguinated and the serum was collected, heated at 56°C for 30 min, and then stored at -20°C until required.

ANTI- θ ASSAY

Cell suspensions of normal thymocytes, hydrocortisone-resistant thymocytes and lymph node cells from CBA/Lac mice were washed twice in medium 199 and made up to 5×10^6 cells per ml. Doubling dilutions of anti- θ _{C3H} serum were made in medium 199 in 5 ml polystyrene tubes. 0.05 ml of cell suspension was added to 0.05 ml of anti- θ _{C3H} dilution, and incubated at 37°C for 45 min. The tubes were then spun down hard ($\approx 1000\text{g}$) for 1 min and the supernatant removed. 0.05 ml of agarose absorbed guinea pig serum (Cohen and Schlesinger, 1970) diluted 1/6 with medium 199 was then added and the tubes shaken. After a further incubation of 30 min at 37°C the tubes were spun down hard again for 1 min. The supernatants were removed and the tubes placed on ice. One drop of 0.2% trypan blue (in saline) was added to each tube in turn, and the cells scored as to whether they excluded (live) or took up

(dead) the dye. Control tubes using normal mouse serum (NMS) instead of anti- θ serum were also set up. Cytotoxicity was calculated using a cytotoxic index (Raff, 1971).

$$\text{Cytotoxic Index} = \frac{\% \text{ kill with anti-}\theta_{\text{C3H}} - \% \text{ kill with NMS}}{100 - \% \text{ kill with NMS}}$$

In another experiment AKR thymus grafts taken from deprived CBA/H.T6T6 mice after hydrocortisone-treatment, were treated with anti- θ AKR serum or normal mouse serum (CBA) and 1/6 agarose absorbed guinea pig serum. After washing, the cells were set up in culture with PHA.

CELL FRACTIONATION

A velocity sedimentation apparatus similar to that described by Miller and Phillips, 1969, was used. The method described was recommended to me by Dr S. Knight and has been used successfully by her (Knight et al., 1973).

A discontinuous albumin gradient was made and all solutions were loaded from a single loading vessel into a 12 cm diameter sedimentation vessel (G.K. Scientific). A stock solution of 2% bovine serum albumin (BSA) was made by adding 5 gm BSA to 100 ml RPMI 1640 medium buffered with Hepes (20 mM) along

with PBS (140 ml) and foetal calf serum (10 ml). Dilutions of this solution were made with PBS. The following solutions were run consecutively into the sedimentation vessel from the loading vessel: PBS (20 ml), 250×10^6 thymus graft cells in 0.2% BSA (20 ml), 0.5% BSA (50 ml), 1% BSA (50 ml), 1.5% BSA (100 ml), 2% BSA (100 ml). The loading time was approximately 45 minutes. The apparatus was left for about $2\frac{1}{2}$ hours at room temperature after which time the cells were collected in 12 ml fractions from the bottom of the sedimentation chamber. The cell fractions were spun down and the cell pellets resuspended in tissue culture medium. After counting each fraction on a Coulter Counter, the counts were adjusted to 2×10^6 cells per ml of medium and 0.5 ml of cell suspension was cultured with 0.5 ml (1×10^6) of syngeneic but chromosomally distinct peripheral blood cells and PHA as previously described.

STATISTICS AND CALCULATIONS

In most experiments the results are expressed as the mean of a given number of observations. In some experiments the means are given plus or minus two standard errors. The significance of some results was assessed using the students "t" test.

RESPONSE OF MOUSE THYMOCYTES TO PHA

There is much evidence showing that normal thymocytes respond poorly to PHA. This has been shown both in humans (McIntyre and Segal, 1966; Claman, 1966; Winkelstein and Craddock, 1967) and experimental animals (Schrek and Batra, 1966; Metcalf, 1966; Statny and Ziff, 1966; Weber, 1966). However, there is also strong evidence indicating that a small minority of thymic lymphocytes can respond to PHA and that these cells are relatively corticosteroid resistant (Blomgren and Svedmyr, 1971; Jacobsson and Blomgren, 1972; Greaves and Janossy, 1972). It is thought that they are located in the medullary areas of the thymus (Ishidate and Metcalf, 1963; Dougherty et al., 1964). Cells in the thymus cortex, which include approximately 90-95% of the total thymus cell population do not seem to exhibit responsiveness to PHA. In the experiments to be reported in this section, it will be shown that mouse thymus graft cells exhibit the same characteristics (as described above) in their response to PHA. Furthermore, a method will be described that allows the mitotic response of normal thymus graft cells to be ascertained even though these cells do not respond on their own in any appreciable way to PHA.

To ascertain the response of normal or hydrocortisone-resistant thymus-graft cells to PHA, thymus grafts were removed from mice which had been grafted with syngeneic neonatal thymuses 52 days previously. These animals had or had not been treated three days previously with 10 mg hydrocortisone sodium succinate. The thymus graft cells were placed in culture with PHA supplemented medium and cultured for three days at 33°C. During the last 16 hours of culture tritiated thymidine was added, and at harvesting the cultures were assessed for the amount of H₃T that the cells had incorporated. Table 1 gives the results. It can be seen that normal thymus graft cells had incorporated very little H₃T whereas hydrocortisone-resistant thymus graft cells had incorporated a substantial amount of H₃T. These results, carried out under the optimal conditions, showed that mouse thymus graft cells behaved in much the same way as mouse thymocytes obtained from thymuses in situ (Blomgren and Svedmyr, 1971).

In the following experiment and many others to be described later on in this thesis, to obtain a mitotic response from normal thymus graft cells it was necessary to co-culture them with normal but cytologically distinct syngeneic peripheral blood lymphocytes. This was done by making cell suspensions

of the relevant lymphocytes up to 2×10^6 nucleated cells per ml. These cell suspensions were mixed in certain proportions and cultured with PHA.

e.g.	Thymocytes		Peripheral blood cells	
	0.7	+	0.3	= 1.0 ml
	0.6	+	0.4	= 1.0 ml
	0.5	+	0.5	= 1.0 ml

The metaphase plates of the harvested cultures were scored, and it could be deduced which dividing cells were of thymus graft or peripheral blood origin. This technique was an extension of the co-culture technique devised by Doenhoff (1971), who cultured two chromosomally distinct syngeneic peripheral blood populations.

The results shown in Figure 2 were obtained when hydrocortisone-resistant thymus graft cells (day 25 post grafting) were mixed with normal peripheral blood cells in the ratios of 1:1, 2:1 or 5:1. It can be seen that the two cell populations did not respond by mitosis equally well over the three cell ratios tested. It seemed that thymus-graft cells responded better than they would have been expected to respond at high thymus graft : peripheral blood cell ratios. Assuming that the response of 1.4:1 (obtained when cells were mixed 1:1) was the correct ratio, then the responses observed at ratios of 2:1 and 5:1 were far higher than could be expected. Similar findings have been found when

normal or hydrocortisone-resistant thymocytes, or normal thymus graft cells have been mixed artificially with normal syngeneic peripheral blood cells. Doenhoff (1971) has shown that when two chromosomally distinct but syngeneic peripheral blood populations were mixed in culture in the same way as described above, they respond equally well over all cell:cell ratios tested. The results shown in Figure 2 show that this was not the case when thymocytes and peripheral blood cells were mixed. One explanation for the lack of equality in racing of these two cell populations could be due to some suppressor effect of thymus cells in culture on peripheral blood lymphocytes at high thymocyte to peripheral blood lymphocyte ratios. The converse of this could be true. Since it has been shown that thymocytes can have a suppressor function in certain situations (Gershon, 1973), it seems likely that thymus cells can suppress in some way the mitotic response of normal peripheral blood lymphocytes (Fig. 2).

Due to the discrepancy seen when thymocytes and syngeneic peripheral blood cells were cultured in vitro with PHA, results to be expressed later on in this thesis take into account only the response of thymus cell populations, and the co-culturing of thymocytes with peripheral blood cells is only used as a method for displaying the thymocyte populations responding, not as a

means of exact quantitation of the number of thymocytes responding.

TABLE 1

Incorporation of H_3T (c.p.m.) into PHA cultures* of Normal and Hydrocortisone-resistant thymus graft cells.

	Thymus graft cells	
	Normal	Hydrocortisone-resistant
2.5 μ g PHA**	565 \pm 49***	10,374 \pm 463

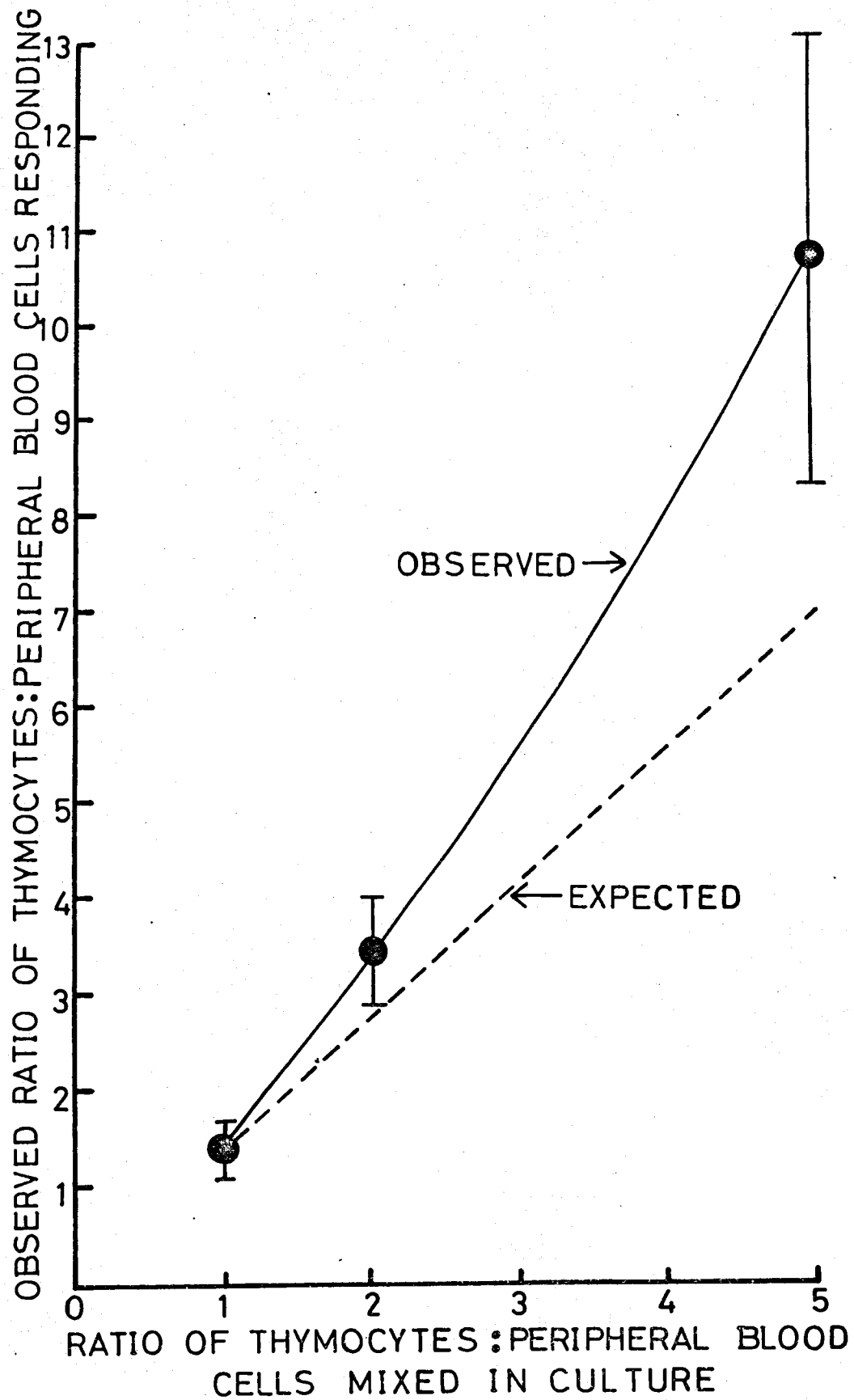
* Cultures contained 2×10^6 nucleated cells in 1 ml. This was found to give the maximal response at 3 days.

** Optimal dose of PHA.

*** Stimulated c.p.m. - non-stimulated c.p.m. \pm 2 standard errors of 3 cultures.

Figure 2

The observed and expected ratios of artificial mixtures of hydrocortisone-resistant thymus graft cells (CBA/H.T6T6) and peripheral blood lymphocytes (CBA/Lac) stimulated with PHA. The circles are means of 7 or 8 samples and the bars represent ± 2 standard errors from the mean. The expected line was drawn assuming that the value obtained when cells were mixed 1:1 was multiplied at higher ratios.



ORIGIN OF PHA-RESPONSIVE CELLS
IN THE MOUSE THYMUS

In the previous section it was shown that a small proportion of thymocytes were capable of responding to PHA. The origins of this minor thymocyte population are obscure. It could derive either from within the thymus itself or be a contaminating peripheral blood cell population residing in the thymus at the time of thymus removal. Evidence will be presented here showing that this population of cells derives from the thymus (via some differentiation pathway within the thymus), and not from a contaminating peripheral blood cell population.

Fourteen male 10 week old CBA/Lac mice were grafted under the kidney capsule with a single CBA/H.T6T6 thymus lobe from 1-2 day old mice. Eighteen days after grafting, when nearly all the mitoses in the thymus are CBA/H.T6T6 in origin but T cells external to the thymus are CBA/Lac (Leuchars et al., 1967), some mice were injected intraperitoneally with 10 mg hydrocortisone sodium succinate. Four days later the injected animals were killed and their thymus grafts were removed and teased apart. One million thymus cells were mixed with an equal number of partially purified peripheral blood lymphocytes from

(CBA/Lac x CBA/H.T6T6) F_1 mice and cultured with PHA for 3 days. Colcemid was added to the cultures 16 hours before harvesting, and cytological preparations were made the following day. Metaphase plates were examined to determine whether the cells were derived from the thymus-grafted mouse (Lac), the thymus graft (T6T6) or the added peripheral blood lymphocytes (F_1). Twenty-one days after grafting, when nearly all the mitoses in the thymus are CBA/Lac in origin (Leuchars et al., 1967), the remaining mice were injected with hydrocortisone and 4 days later the same cultural procedures were carried out on their thymus grafts.

Analysis of the cytological preparations gave the results shown in Table 2. When one examines the origin of the PHA-responsive cells in thymus grafts from hydrocortisone-treated animals (ignoring F_1 peripheral blood mitoses), it can be seen that approximately 2% were derived from the host animal (Lac) and approximately 98% were derived from the thymus graft (T6T6), indicating that the PHA-responsive hydrocortisone-resistant cells in the thymus grafts are not blood-borne T cells.

Further interest accrues to the 21 day result as, at this time after grafting, most of the cells dividing in the thymus graft in vivo in otherwise untreated animals are of host (Lac) type

(Leuchars et al., 1967). At this time the PHA-responsive cells are still of thymus graft donor type suggesting that, following mitotic activity, cells persist in the thymus for an unknown length of time after dividing in vivo in the thymus.

The residual cells in the thymus after hydrocortisone treatment, like peripheral blood cells, have a low grade theta (θ)-positivity, and both cell populations respond to PHA (Raff and Owen, 1971; Raff, 1971b). Nevertheless the residual cells and peripheral blood cells are not apparently part of the same population, and the residual cells might be best thought of as having undergone thymic processing but not yet having been released from the thymus into the peripheral T cell pool.

Weber has shown that PHA-responsive cells in the pig thymus reside in the medulla rather than the cortex (Weber, 1966a, 1966b) and has also concluded that less than 1% of these cells were contaminating blood-borne PHA-responsive cells (Weber, 1970). Thus there is strong agreement between the results presented in this section, and the conclusions Weber drew from his experiments.

Since it is thought that corticosteroids can cause involution of the thymic cortex while leaving the medulla relatively unaffected (Warner, 1964), it thus seems likely that the differentiation of cells within the thymus involves a hydrocortisone-sensitive mitotic

stage in the cortex. Such cells are not PHA-responsive. Later these cells are less sensitive to hydrocortisone, non-mitosing, they can react to PHA and are found in the medulla before some at least are liberated into the circulation.

TABLE 2

Origin of PHA-responsive cells in mouse thymus grafts

	Day 18 after thymus grafting		Day 21 after thymus grafting	
	Thymus grafted mouse in origin (Lac)	Thymus graft in origin (T6T6)	Thymus grafted mouse in origin (Lac)	Thymus graft in origin (T6T6)
No. of metaphase plates examined	17	721	27	1124
% Lac	2.3		2.3	
% T6T6	97.7		97.7	

4 mice examined at each time.

PERSISTENCE OF PHA-RESPONSIVE CELLS IN

MOUSE THYMUS GRAFTS

It has been shown that the hydrocortisone-resistant cells in the mouse thymus that respond to PHA are not part of the recirculating pool of T cells but are derived from the thymus graft, and the notion has built up that the corticosteroid-resistant cell population can be equated with a mature cell population in the thymus which is ready to leave the organ and become part of the peripheral T-cell pool. Here it will be demonstrated that under certain experimental circumstances, the PHA-responsive cell population in the thymus largely consists of cells which seem never to leave the thymus and which are therefore probably not to be equated with peripheral T cells.

Adult male CBA/Lac mice had implanted under the kidney capsule single thymus lobes from 1-2 day old CBA/H.T6T6 donors. At various times later the thymus grafts were removed and the cells they contained were washed and co-cultured with (CBA/Lac x CBA/H.T6T6) F_1 peripheral blood lymphocytes. On the evening of the second day colcemid was added to the cultures and on the third day the cells were prepared for cytological analysis. Three kinds of cells were distinguishable in the cultures -

CBA/H.T6T6 cells, native to the thymus graft; CBA/Lac host cells, deriving from the thymus graft recipient; and (CBA/Lac x CBA/H.T6T6) F_1 cells, from the peripheral blood addition to the cultures. Only cells of known thymus-graft origin (T6T6 and Lac) are recorded. It can be seen from Figure 3 that the grafts at all times tested contain a majority of PHA-responsive native cells (T6T6), even at 217 days after thymus grafting. This result is independent of the presence or absence of a thymus in situ in the recipient mouse.

It could be argued that the finding of these native cells is because the recipient mouse has a full complement of T-cells and that in some way this prevents the emergence of mature T cells from the thymus graft. Accordingly the experiment was repeated using as recipients thymectomized, irradiated, bone marrow injected adult mice. In this situation it might be supposed that the full potential of the thymus graft could be exercised, the recipient having very few T cells (Doenhoff et al., 1970b). It can be seen from Figure 4 that an even higher percentage of native PHA-responsive cells was found. Thus the existence of a persistent PHA-responsive population was not affected by the lymphoid status of the host mice.

One criticism that could be levelled against these findings

is that the day 3 in vitro response of these persistent cells to PHA is not a true estimate of their responsiveness, and that by looking earlier or later at the in vitro PHA response, different results might be obtained. Thus an experiment was set up to see if there was any difference in the percentage of persistent PHA-responsive cells responding, when duplicate cell cultures were harvested on successive days from 1-5. Figure 5 gives the results. It can be seen in the two experiments shown in Figure 5 that there was no difference in the percentage of persistent cells responding when the cultures were harvested from days 1-4 or from days 3-5 (different thymus graft populations), and that a high percentage of persistent PHA-responsive cells was observed at each time studied. These results do not take into account the fact that few cells were seen mitosing at day 1 and 5, although the percentages obtained on these days are comparable with those obtained when a greater number of mitoses were visible (days 2-4). Thus, unlike the results obtained by Piguet and Vassalli (1972) who showed a rise in one mitotic population but a decrease in the other during the course of the PHA response of spleen cells from mouse radiation chimaeras, these results show no variation over the whole time course studied.

Two points should be made concerning the results

presented in this section. Firstly they indicate that there is a persistent population of cells in the thymus which respond to PHA and which probably should not be equated with T-cells external to the thymus despite certain behavioural similarities, and secondly that in all grafts studied some cells were found from the host, indicating perhaps that this minority cell population could indeed be a mature cell population about to leave the thymus (Borum, 1968).

Figure 3

The percentage of donor-type (T6T6) PHA-responsive cells in CBA/H.T6T6 thymus grafts from normal (●) or adult thymectomized (▲) CBA/Lac mice at various times after grafting. Minimum of 3 mice per point.

An average of 121 mitotic figures of thymus graft origin were scored per point.

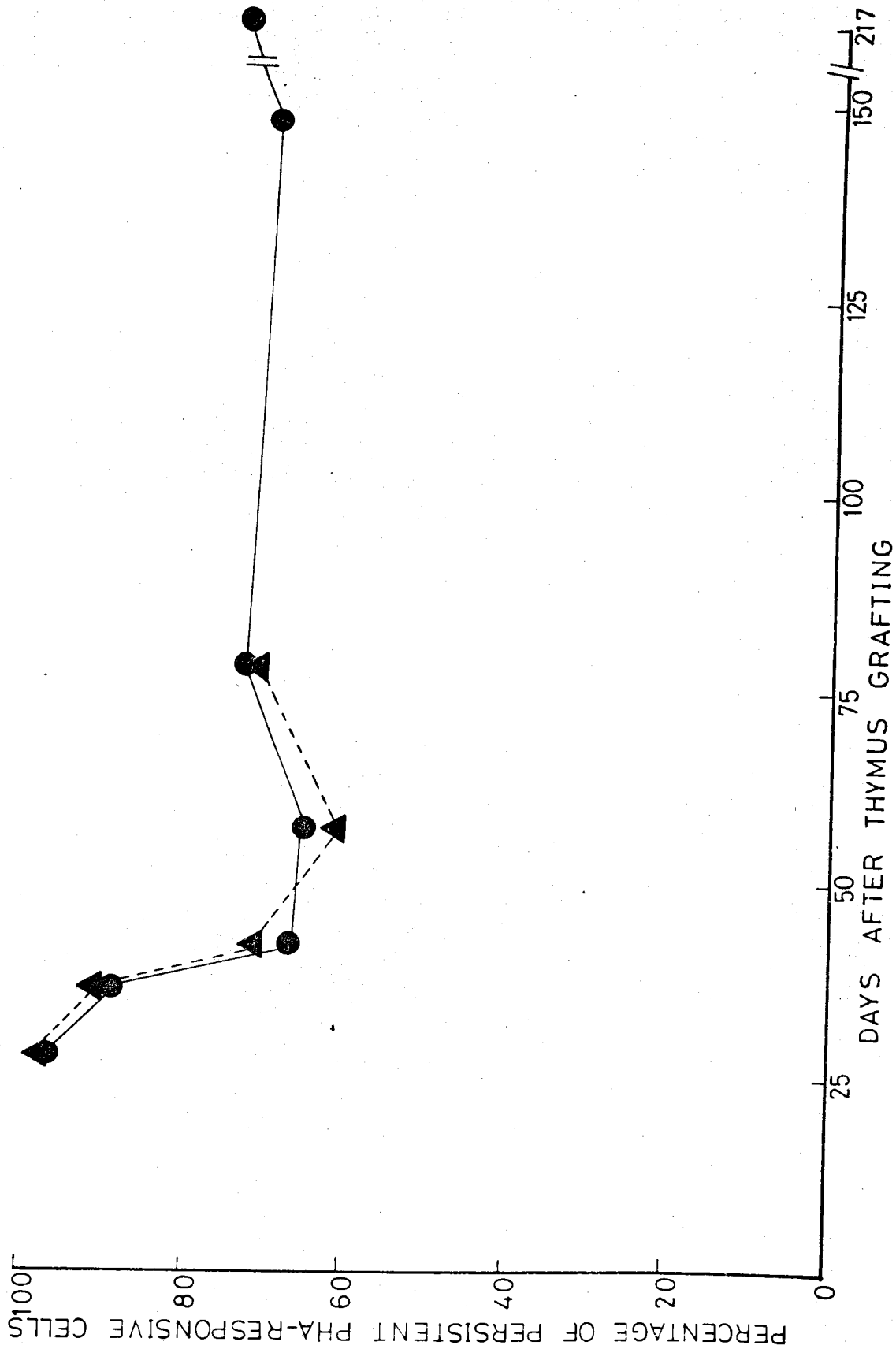


Figure 4

The percentage of donor-type (T6T6) PHA-responsive cells in CBA/H.T6T6 thymus grafts from thymectomized, irradiated CBA/Lac mice injected with CBA/Lac bone marrow, at various times after grafting.

Minimum of 3 mice per point.

An average of 123 mitotic figures of thymus graft origin were scored per point.

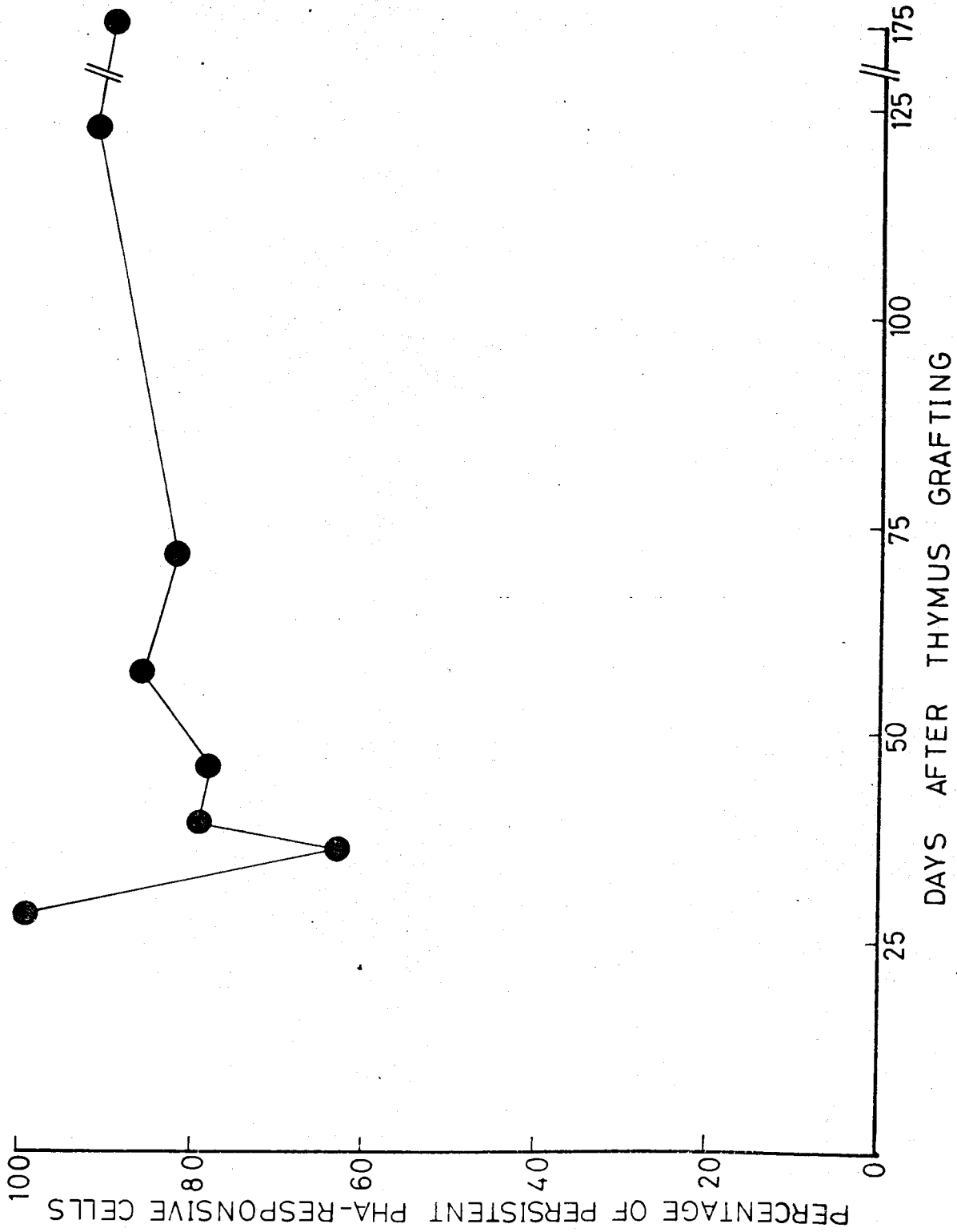
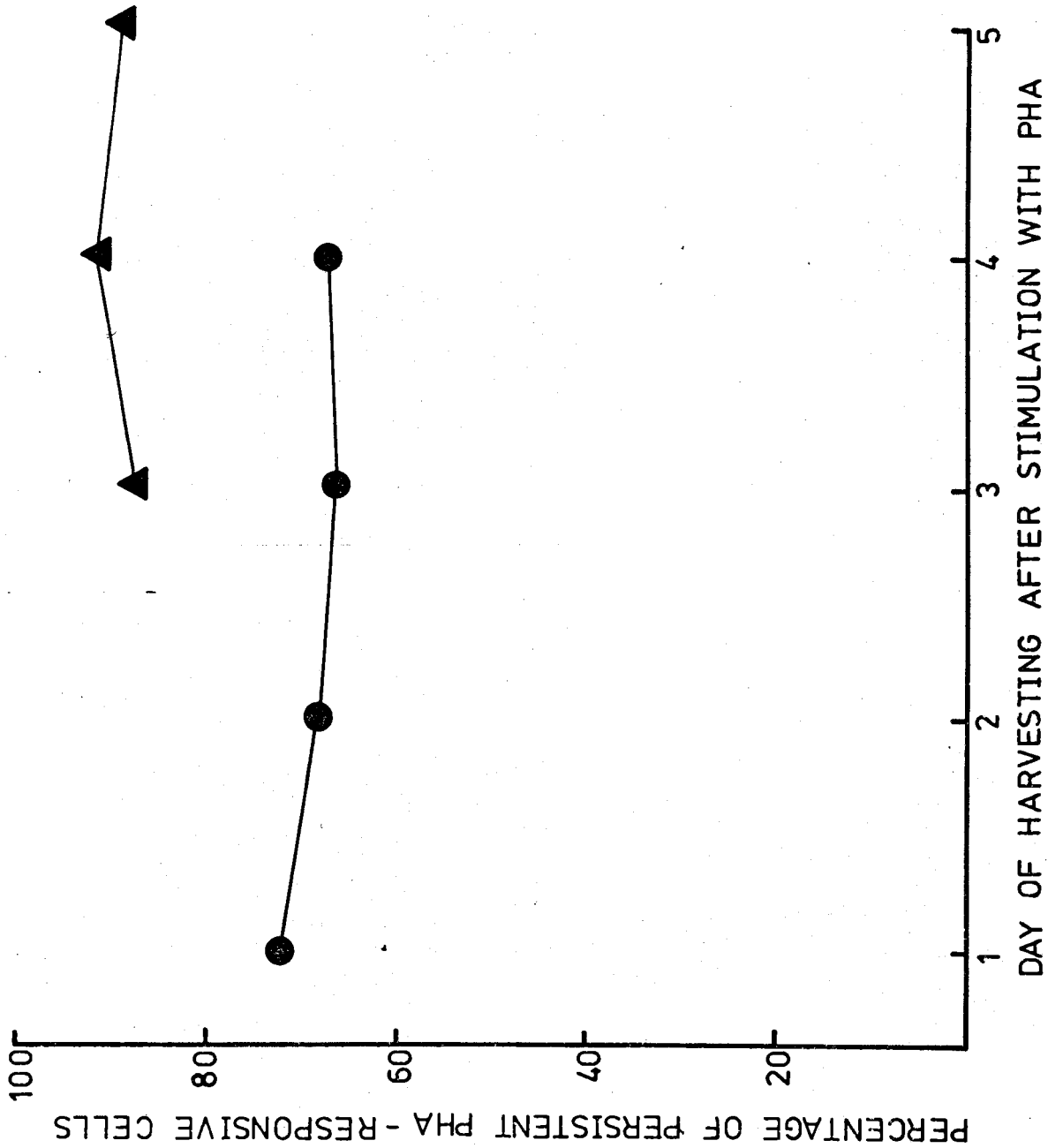


Figure 5

A time course study of the percentage of persistent PHA-responsive cells from thymus grafts removed from normal mice 49 (●) or 65 (▲) days after thymus grafting.

Each point represents the mean of two cultures and an average of 63 mitotic figures of thymus graft origin were scored per point.

(day 1, 20; day 2, 58; day 3, 233; day 4, 138; day 5, 43)



RESPONSE OF NATIVE THYMUS GRAFT CELLS
TO OTHER MITOGENS

In the previous section it was shown that a high percentage of cells from mouse thymus grafts responding by mitosis to the mitogen PHA had the chromosome marker of the thymus graft donor, even at late times after grafting. It has also been shown that other mitogens can activate normal and/or cortisone-resistant thymocytes (Greaves and Janossy, 1972). With these facts in mind, experiments were set up to see if the persistent thymocyte population would respond to other supposed T cell mitogens, and also to see whether it would respond to a mitogen that only activates B cells (reviewed by Greaves, Owen and Raff, 1973).

Thymus grafts were removed from normal animals (Lac) which had implanted at least 70 days previously a syngeneic but chromosomally distinct thymus graft (T6T6). The washed graft thymocytes were placed in culture with (Lac x T6T6) F_1 peripheral blood cells, and one of a number of mitogens known to activate T cells. The results of two such experiments are shown in Table 3 (F_1 peripheral blood cells excluded). It can be seen that with all four mitogens tested the persistent population was expressed although to differing degrees. The order of

responsiveness appeared to be PHA > Con A > PWM > SEB. The reason for these differences are not clear. It would appear that Con A and PWM can activate normal thymocytes to a greater extent than PHA (Greaves and Janossy, 1972), but this alone would not explain the results found, since Con A causes a greater proliferation of normal thymocytes than PWM. Yet the percentage of persistent cells is higher with Con A, the opposite of what would be expected if Con A was activating a larger proportion of cortical thymocytes than PWM. Another factor that might have some bearing on the results is that PWM (Greaves and Janossy, 1972) and SEB (W. Adler, personal communication) can activate both T and B cells, but since thymocyte populations contain usually less than 2% B cells (Vitetta, Uhr and Boyse, 1973) it seems unlikely that this small percentage of B cells could cause the discrepancies seen between the different mitogens. The third possibility is that the persistent and non-persistent PHA-responsive populations demonstrated previously can respond differentially to different T cell mitogens.

To test whether the persistent thymus-graft cell population could respond to a purely B-cell mitogen (LPS), hydrocortisone-resistant thymus graft cells were cultured on

their own with LPS. There was no detectable response of these cells to this mitogen. This result on its own was not considered to be good enough evidence that thymocytes do not respond to LPS since Doenhoff (personal communication) has shown that although normal peripheral blood lymphocytes will not respond to LPS on their own they can be made to respond when co-cultured with normal spleen cells, i.e. in the presence of a population that can itself respond to LPS (Greaves and Janossy, 1972). Bearing this in mind, hydrocortisone-treated thymus graft cells were co-cultured in equal numbers with normal spleen cells, and stimulated with either PHA or LPS. The results of two such experiments can be seen in Table 4. It will be noted that when PHA was the stimulant, the two cell populations responded almost equally well yet when LPS was used to stimulate the cultures, no cells of thymus-graft type could be seen to be responding. This was taken as evidence that thymus graft cells do not respond to LPS, and that the contamination of thymocyte populations by B-cells was so slight as not to be observable in these experiments.

In conclusion it should be stressed that the persistent thymus graft population was activated by PHA, Con A, PWM and SEB (although in different proportions), mitogens which have been shown to activate T cells, but not by LPS which is a known B cell mitogen.

TABLE 3

Percentage of cells of native thymus graft origin (persistent)
responding to various mitogens.

	<u>Experiment 1 *</u> (49 days post grafting)	<u>Experiment 2 *</u> (70 days post grafting)
PHA	90 **	70
Con A	82	56
PWM	67	N.D.
SEB	42	48

* 3 thymus grafts examined in each experiment.

** 50 mitotic figures scored per culture.

TABLE 4

Mitotic response of hydrocortisone-treated thymus graft cells to PHA or E. coli LPS.

	Percent thymus graft population*	Percent spleen population
<u>Experiment 1</u>		
PHA	44**	56
LPS	0	100
<u>Experiment 2</u>		
PHA	47	53
LPS	0	100

* A pool of thymus graft and spleen cells were used in each experiment.

** Between 70 and 100 cells were scored per culture.

EFFECT OF HYDROCORTISONE AND ANTI-LYMPHOCYTE
SERUM ON PERSISTENT PHA-RESPONSIVE CELLS
IN MOUSE THYMUS GRAFTS

(a) Hydrocortisone

The fact that corticosteroids can cause destruction of the thymus cortex but leave the medulla relatively unaffected has been amply demonstrated (Warner, 1964; Dougherty et al., 1964; Ishidate and Metcalf, 1963). It was important with this in mind to see if hydrocortisone treatment of animals bearing thymus grafts had any effect on the persistent PHA-responsive cells that have been shown to exist in normal thymus grafts (vide supra). In the first experiment, normal CBA/Lac mice bearing CBA/H.T6T6 thymus grafts under their kidney capsules were injected intraperitoneally, seventy days after grafting, with 10 mg hydrocortisone sodium succinate. Three days later cells were collected from the grafts and placed in culture with (CBA/Lac x CBA/H.T6T6) F₁ peripheral blood lymphocytes. Cytological analysis after three days in culture revealed that 89% of the PHA-responsive cells of graft origin were CBA/H.T6T6 (i.e. native to the graft). In a comparable control group not treated with hydrocortisone, the CBA/H.T6T6 cells constituted 86% of the PHA-responsive cells of graft origin. Thus there

seems little doubt that the hydrocortisone-resistant cell population contains the same kind of persistent cells as are present in the untreated thymus graft.

In a further experiment the long term effects of hydrocortisone on the persistent PHA-responsive population were ascertained. In this experiment normal CBA/Lac mice grafted 66 days previously with a CBA/H.T6T6 thymus graft were injected intraperitoneally with 10 mg hydrocortisone sodium succinate (control mice received saline), and at various days after injection, animals were killed, their thymus grafts removed, washed twice in medium 199, counted and cultured with (CBA/Lac x CBA/H.T6T6) F_1 peripheral blood lymphocytes and PHA. The results shown in Table 5 give the counts of nucleated cells obtained from these grafts, and it can be seen that the counts were similar between the two groups apart from the five day figure which was depressed significantly in the hydrocortisone-treated grafts ($p < 0.05$). From other observations it would seem that after this particular dose of hydrocortisone the thymus had already started regenerating at day 5, and that at maximum involution (day 2-3) the thymus is reduced in weight to approximately 10% of control values. Table 6 shows the effect of hydrocortisone treatment on the percentage of persistent-PHA-responsive cells in these same thymus graft cell populations.

It can be seen that at most times after treatment there is no significant difference between hydrocortisone or saline treated thymus grafts. The only time when the results are significantly different is 9 days ($p < 0.02$). The reason for the higher percentage in hydrocortisone-treated grafts is obscure but it could be explained by assuming that after hydrocortisone treatment the persistent PHA-responsive population remains in the thymus graft whereas the non-persistent population (some cells of which were probably about to leave anyway (Borum, 1968)) leaves the thymus graft. Before this non-persistent population could be regenerated after hydrocortisone treatment there would be a period when the percentage of persistent cells would be elevated. The main conclusion from all of these findings is that the persistent PHA-responsive population found in non-treated thymus grafts is probably the same as that found in hydrocortisone-treated grafts. This would tend to localize this population in the thymic medulla rather than the thymic cortex, since the cells in the latter region are extremely steroid sensitive.

(b) ALS

The immunosuppressive effects of ALS have been attributed to the selective depletion of recirculating T lymphocytes (reviewed by Lance, Medawar and Taub, 1973). With this in mind it might be assumed that lymphoid tissues such as the thymus, which do not seem to bear significant numbers of blood-borne lymphocytes (vide supra), would be spared the effects of ALS. Experiments which would tend to support this assumption were carried out by Lance (1969), who showed that the labelled immunoglobulin (IgG0 fraction of ALS penetrated the thymus in only very small amounts, and by Raviola and Karnovsky (1972) who presented evidence for a blood-thymus barrier to certain antigens. Quite apart from having a debilitating effect on the thymus, it has been shown quite recently that ALS can enhance the GvH potential of thymocytes (Cantor and Asofsky, 1973). With all these studies in mind, experiments were performed to see what effect, if any, ALS has on the persistent PHA-responsive cells found in mouse thymus grafts. The ALS preparation used, and its suppressive effect on circulating T-cells have been described in the Materials and Methods section. In the experiment to be described here, normal CBA/Lac mice grafted 66 days previously with a CBA/H.T6T6 thymus graft were injected

subcutaneously two times (separated by 2 days) with 0.5 ml ALS (control mice received saline), and at various days after the second injection animals were killed, their thymus grafts removed, washed twice in medium 199, counted and cultured with (CBA/Lac x CBA/H.T6T6) F_1 peripheral blood lymphocytes and PHA. The results shown in Table 5 give the counts of nucleated cells obtained from these grafts. At no time were the counts between the two groups significantly different, although nine days after ALS treatment there was an increase in cell count in the ALS treated group ($0.2 > p > 0.1$). These results agree well with those of Cantor and Asofsky (1973) who also showed no difference in cell counts up to 21 days after ALS treatment. Table 6 shows the effect of ALS treatment on the percentage of persistent PHA-responsive cells in these same thymus graft cell populations. The results show that there was no difference between the ALS-treated and control groups even as late as 153 days after treatment. Cantor and Asofsky (1973) showed that the GvH potential of thymocytes was increased for a long period after ALS treatment, and although in the experiment shown in Table 6 studies on the immunological function of thymus graft cells were not carried out, it might have been expected that there would be a disturbance in the percentage of persistent PHA-

responsive cells. That this is not the case is obvious but the reasons for it are obscure. The most likely explanation for this lack of agreement is that Cantor and Asofsky used lower amounts of ALS to get the effects they described (0.1 x 0.25 ml) whereas in the experiment described above two doses of 0.5 ml ALS were used.

One reason why this ALS experiment was performed was to test a hypothesis that the persistent PHA-responsive cell population was a population of cells in the thymus that could be readily mobilized in the event of any catastrophe afflicting the T-cell population in the periphery. This hypothesis would predict that after depletion of the peripheral T cell population by ALS, the cells in the thymus would be able to leave the thymus and replace those cells destroyed by ALS. As a consequence of this, the percentage of cells that were known to be persistent would be decreased and this would be reflected in the percentage of persistent PHA-responsive cells found in thymus grafts after ALS treatment. Since the dose of ALS used did reduce the peripheral T cell pool to 3.7% of control values but the percentage of persistent PHA-responsive cells was unchanged at all times studied, it would seem that this hypothesis had no validity.

TABLE 5

The nucleated cell counts of thymus grafts * taken at various times after hydrocortisone, ALS or saline treatment.

Day after treatment	<u>Cell counts x 10⁻⁶ per thymus graft</u>		
	Hydrocortisone	Saline	ALS
5	8.6	^{**} ←p<0.05→ 24.4	25.5
9	23.4	25.3	←p>0.1→36.1
13	24.1	21.9	23.1
20	25.7	18.5	21.3
27	25.2	18.7	18.3
153	12.7	13.0	12.2

* Mice grafted 66 days before treatment.

** No other figures were significantly different at any particular day after treatment.

Minimum of 3 mice per group.

TABLE 6

Percentage of persistent PHA-responsive cells in thymus grafts*
taken at various times after hydrocortisone, ALS or saline
treatment.

Day after treatment	Hydrocortisone	Saline	ALS
5	88.4	85.5	83.9
9	91.7	80.6	80.9
13	90.9	83.6	83.4
20	79.2	84.5	78.9
27	75.0	75.9	78.8
153	75.9	78.7	75.8

* Mice grafted 66 days before treatment.

** No other figures were significantly different at
any particular day after treatment.

Minimum of 3 mice per group.

Average of 96 mitoses scored per group.

PERSISTENT PHA-RESPONSIVE CELLS IN ALLOGENEIC
THYMUS GRAFTS

Syngeneic thymus grafts when implanted into deprived CBA mice survive indefinitely and have been shown to liberate T cells which incorporate into the peripheral T cell pool (Davies et al., 1971; Doenhoff et al., 1970b). Varying the genotype of the thymus graft only slightly, has little effect on both survival (Leuchars et al., 1970) and incorporation of cells into the peripheral T cell pool (Doenhoff, 1970). When grafts which differ at the H-2 locus have been implanted into deprived CBA mice on the same day as deprivation, they often fail to survive (Dukor et al., 1965; Leuchars, 1966), yet variation of haematopoietic reconstituent, or time of grafting allows a much larger number of grafts to survive (Leuchars et al., 1970). Whether these H-2 different grafts that survive function in the same way as H-2 similar grafts is a matter of some speculation at the moment. With these experiments in mind it was thought worthwhile to see if the persistent PHA-responsive population found in syngeneic thymus grafts, was present in allogeneic thymus grafts.

In the first series of experiments AKR (H-2^k) newborn thymuses were implanted under the kidney capsule of adult

thymectomized, irradiated (850r) and bone marrow treated (CBA/H.T6T6 5×10^6 BM cells i.v.) CBA/H.T6T6 (H-2^k) mice, 3 to 4 hours after irradiation. In all three experiments graft survival was 100%, even when judged 406 days after grafting. At various times after grafting, the grafts were removed, teased, the cells washed twice in medium 199 and cultured with (CBA/Lac x CBA/H.T6T6)F₁ peripheral blood and PHA. In this system cells of native thymus graft (AKR) origin would have no minute chromosome markers whereas cells of the host and bone marrow would have two. Analysis of the metaphase plates of these cultures at various times after thymus grafting revealed in Table 7 that cells of native thymus graft (AKR) origin were present in quite high percentages. The results were similar to those seen with syngeneic thymus grafts apart from being lower and more variable. Thus there are similarities between completely syngeneic and slightly allogeneic thymus grafts in their ability to produce PHA-responsive cells that persist in the thymus graft. This was not altogether unexpected since graft survival in deprived hosts is the same (100%). Also the change-over from one cortical population to another is much the same in animals bearing slightly allogeneic thymus grafts (Owen and Raff, 1970 - shown by changeover in theta (θ)) and those bearing

syngeneic grafts (Leuchars et al., 1967 - shown by chromosome markers), and liberation of T-cells into the circulation is comparable with both types of graft (Doenhoff, 1970).

In the second experiment deprived CBA/H.T6T6 (H-2^k) mice were grafted under their kidney capsule with a newborn BALB/c (H-2^d) thymus graft ten days after irradiation and bone marrow (CBA/H.T6T6). These thymus grafts are not usually accepted when implanted on the same day as deprivation but are accepted if grafted 10 days after irradiation and bone marrow therapy (Leuchars et al., 1970; Aird, 1971). Grafts for culture were treated in the same way as AKR grafts, and the marker chromosome situation was similar. Table 8 shows the results. Although only two times were studied it can be seen that some cells of native (BALB/c) thymus graft type were seen responding to PHA at both times. The percentage of persistent cells was much lower than when H-2 similar grafts were implanted, but this could be due to the effect of grafting at 10 days post deprivation, rather than to an H-2 dissimilarity between the graft and recipient. No full explanation of these particular results can be offered but further experiments, especially using foetal liver as the haematopoietic reconstituent, might show if and/or why this reduction of persistent PHA-responsive cells occurs.

In conclusion it should be stated that persistent PHA-responsive cells can be demonstrated in allogeneic H-2 similar thymus grafts (AKR) as well as in H-2 dissimilar thymus grafts (BALB/c), but the percentages in both cases are lower than found in completely syngeneic thymus grafts.

TABLE 7

The presence of persistent PHA-responsive cells in AKR thymus grafts * removed from CBA/H.T6T6 mice at various times after grafting.

Percent mitoses of thymus graft cells**

	Days after grafting	No. of grafts examined	AKR (native)	CBA/H.T6T6
Expt. 1	60	3	70.9	29.1
	89	4	56.7	43.3
Expt. 2	75	Cells pooled from 3 grafts	26.0	74.0
Expt. 3	406	2	63.1	36.9

* Grafts implanted on the same day as irradiation and bone marrow therapy.

** Average of 46 cells scored per culture.

TABLE 8

The presence of persistent PHA-responsive cells in BALB/c thymus grafts * removed from CBA/H.T6T6 mice at various times after grafting.

Days after grafting	<u>Percent mitoses of thymus graft cells***</u>	
	BALB/c (native)	CBA/H.T6T6
60 **	15	85
89	6	94

* Grafts implanted ten days after irradiation and bone marrow therapy.

** 3 mice per group.

*** Average of 38 cells scored per culture.

ANTI-THETA(θ)-SENSITIVITY OF PERSISTENT
PHA-RESPONSIVE CELLS FROM MOUSE THYMUS GRAFTS

The theta (θ) alloantigen was first described by Reif and Allen (1963, 1964). Since that time it has been shown to be a marker for lymphocytes of the T-cell series in mice (reviewed by Raff, 1971b). Thus nearly all lymphoid cells in the thymus are θ -positive as are T-cells in the spleen, lymph nodes, blood, etc.. In the thymus over 98% of the lymphocytes can be killed by anti- θ serum and complement, but there does however remain a small population (<2%) which is insensitive to anti- θ serum, as well as the stromal and reticular cells that make up the network of the thymus. Also in the mouse it has been shown that cells responding by mitosis to PHA are T cells (Doenhoff et al., 1970a), and that PHA-responsive cells are θ -positive (Owen, Hunter and Raff, 1971).

Bearing in mind these experiments it was thought unlikely that the persistent PHA-responsive cells found in thymus grafts would be θ -negative. Thus experiments were undertaken to show that normal and hydrocortisone-resistant thymus graft cells had similar amounts of θ antigen to the corresponding cells from the thymus in situ, and a formal proof was sought to show that persistent PHA-responsive cells were indeed θ -positive.

In the first experiment cell suspensions were made from normal CBA thymus grafts, hydrocortisone-treated CBA thymus grafts or normal CBA lymph nodes. These cell suspensions were mixed with doubling dilutions of AKR anti- θ_{C3H} antisera and complement as described in the Materials and Methods section. The viability of the cell suspensions was ascertained by the trypan blue dye exclusion test, and cytotoxic indices were calculated. Figure 6 shows the results of two such assays. It can be seen that normal thymus graft cells (top) were killed by lower concentrations of anti- θ than were hydrocortisone-treated thymus graft cells (middle) or normal lymph node cells (bottom). The latter two cell populations were killed by much the same dilutions of anti- θ serum. Three points emerge from this experiment. Firstly, normal thymocytes are more sensitive to the killing effects of anti- θ plus complement than are hydrocortisone-treated thymocytes. Secondly, hydrocortisone-treated thymocytes are killed by much the same concentrations of anti- θ as are normal lymph node cells (Raff and Owen, 1971; Leckband and Boyse, 1971). Thus thymus graft cell populations would appear to have similar amounts of θ as do cells taken from the thymus in situ. Thirdly, it will be noted that in both thymus graft populations, there were some cells that were θ -negative,

and it was conceivable that the persistent PHA-responsive cell population could derive from this θ -negative population.

In the second experiment, advantage was taken of the fact that AKR thymus grafts in deprived CBA/H.T6T6 mice survive and produce persistent PHA-responsive cells (vide supra). In this situation T-cells of the thymus graft donor (AKR) would bear the θ_{AKR} antigen, whereas T-cells of the thymus graft recipient would bear the θ_{C3H} antigen. If treatment of these thymus graft cells with anti- θ_{AKR} serum abolished the persistent PHA-responsive population then this would be good evidence that this cell population was θ -positive. Thus cells were removed from hydrocortisone-treated AKR thymus grafts, treated with CBA anti- θ_{AKR} or normal CBA serum, and cultured with PHA (flow sheet shown in Table 9). The result of this experiment is shown in Table 10. It can be seen that treatment of these thymus graft cells with CBA anti- θ_{AKR} serum completely abolished the appearance of mitosing AKR cells, whereas treatment with normal CBA serum had no such effect. Thus it was shown that the PHA-responsive cell population found in allogeneic thymus grafts was θ -positive.

Figure 6

Cytotoxicity of normal thymus graft cells(top),
hydrocortisone-treated thymus graft cells (middle), and
normal lymph node cells (bottom) after incubation with
anti- θ serum and complement.

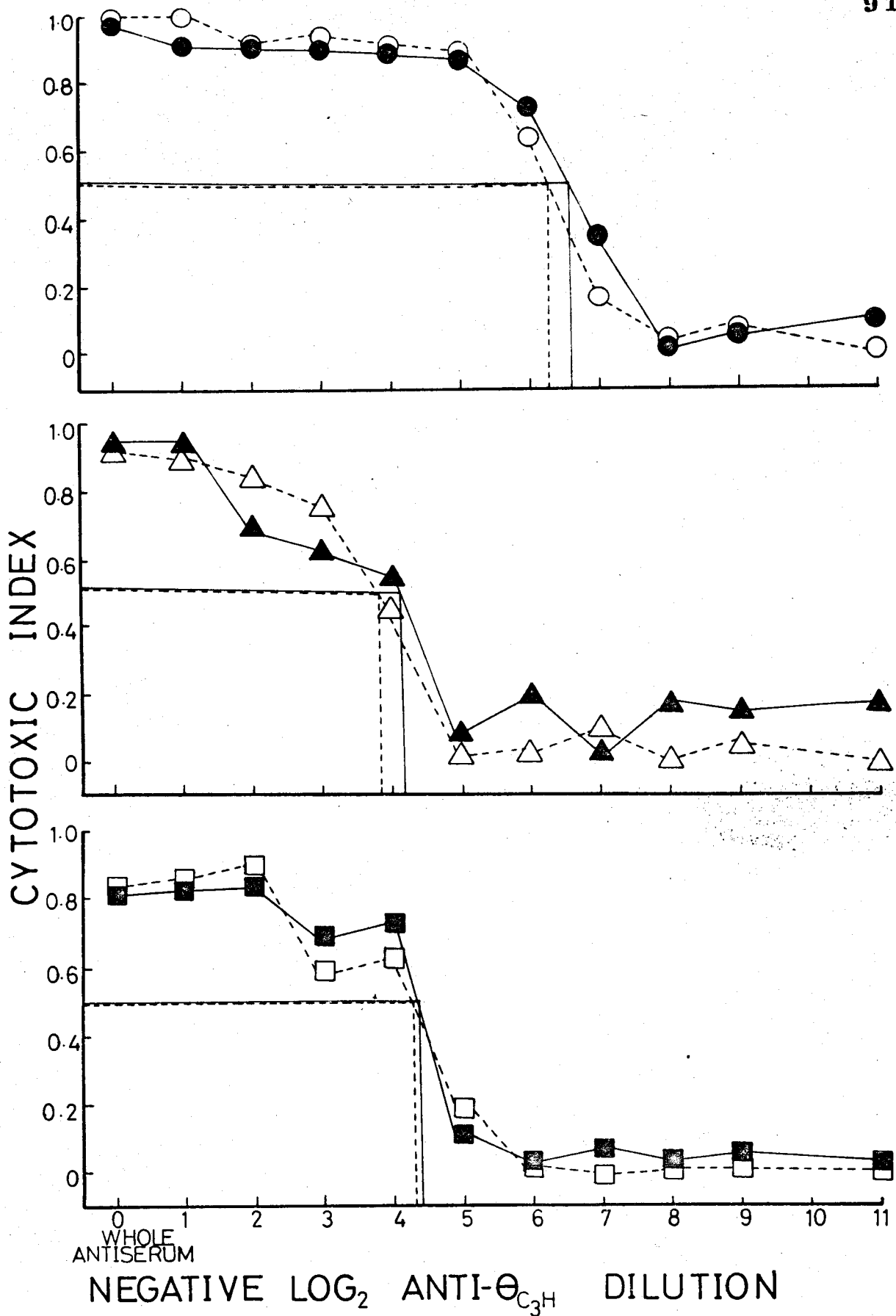


TABLE 9

Flow sheet of experiment showing persistent PHA-responsive cells are θ -positive.

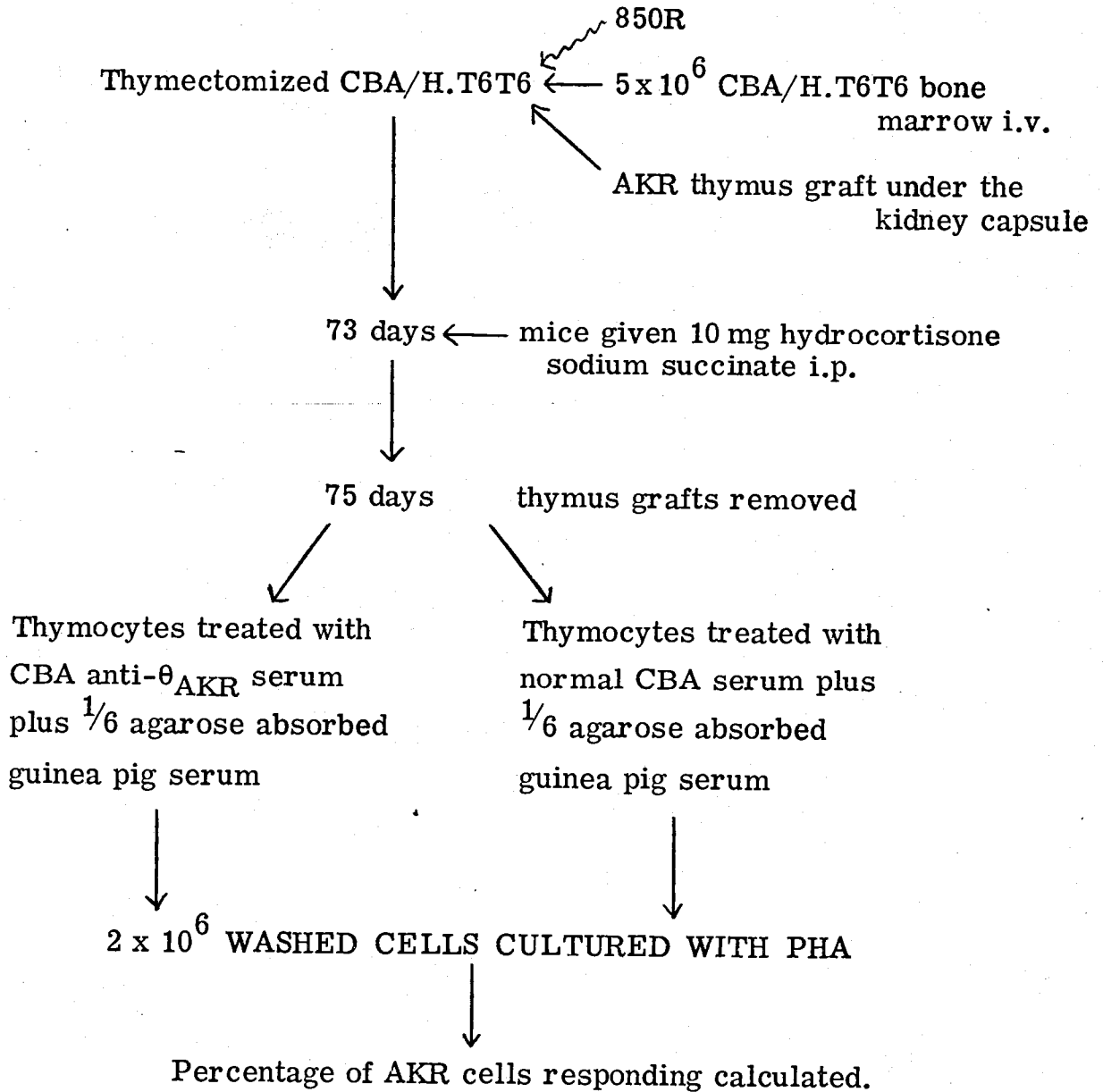


TABLE 10

θ -Positivity of persistent PHA-responsive thymus graft cells*

	CBA anti- θ_{AKR} + GPC treated cells	Normal CBA serum + GPC treated cells
Percentage of AKR cells responding	0**	26
Percentage inhibition of AKR cells by anti- θ_{AKR}	100	

* Pool of cells from 3 grafts.

** 100 cells counted.

SEDIMENTATION OF MOUSE THYMUS GRAFT CELLS

Evidence has been presented above that suggests there is heterogeneity in the cell populations that can respond to PHA in mouse thymus grafts. Although most of the cells that respond have the karyotype of the original thymus graft donor, there was always an appreciable percentage of cells responding that had the recipient karyotype. The cell population showing the recipient karyotype could consist of cells that persist in the thymus but do not possess the "persistent" karyotype, or of cells that are about to leave the thymus (Borum, 1968). It will be shown in later sections that some of these "non-persistent" PHA-responsive cells do seem to have different functional characteristics to the "persistent" cell population. In this section however, experiments were performed to see if there could be demonstrated any physical differences between the "persistent" and "non-persistent" PHA-responsive thymus graft cell populations.

A velocity sedimentation system was used to separate thymocytes (Knight, Newey and Ling, 1973). The method used (explained more fully in the Material and Methods section) allowed thymus graft cells to sediment in a chamber at room temperature and a pressure of 1G. After allowing cells to sediment for about

2½ hours, various cell fractions were collected from the bottom of the gradient, counted, and stimulated with PHA. This method is a modification of that of Miller and Phillips (1969), and these workers suggested that cell separation was due mainly to cell size. Thus large cells would tend to sediment faster than small cells and consequently be found in the earlier fractions collected.

The results of the first experiment (Figure 7) show what happens when 200×10^6 thymus graft cells (49 days post grafting) were sedimented by the above method. In this particular experiment only 63% of the cells put on the gradient could be recovered, but the viability remained at greater than 90% in all fractions tested throughout the gradient. The viability of the starting population was >95%. It can be seen that although the majority of cells separate in fractions 9-16, the cells that respond to PHA by incorporation of tritiated thymidine, were found in lower fractions (1/4 → 11). These results compare well with those of Knight, Newey and Ling (1973) who separated rat thymocytes on similar gradients. They too showed PHA and Con A reactivity in the lower fractions whereas most of the cells were found higher up the gradient. In the experiment reported here there was a bimodality of cell counts in the higher fractions whereas in another experiment no such bimodality could be

demonstrated (see Figure 8). Whether there is a real bimodality is of course unproved, although it must be noted that Knight and her coworkers showed some bimodality in their experiments on rat thymocytes.

With the knowledge that thymus graft cells would separate satisfactorily on these gradients, experiments were set up to determine if the "persistent" cell population could be separated from the "non-persistent" recipient cell population. In the first experiment (Fig. 8), 153×10^6 thymus graft cells (showing a viability of $> 95\%$) taken from animals (CBA/Lac) that were grafted subcutaneously 136 days previously with 14 syngeneic but chromosomally marked newborn thymuses (CBA/H.T6T6), were placed on the gradient and sedimented for $2\frac{1}{2}$ hours at room temperature. Fractions were collected, washed and counted. One million fractionated thymus graft cells were mixed with one million (Lac x T6T6) F_1 peripheral blood lymphocytes and cultured with PHA for 3 days. In this particular experiment cell recovery was 51% of those cells placed on the gradient, but viability in all fractions tested throughout the gradient was greater than 88%.

After 3 days in culture (colcemid being added during the last 5 hours of culture) the cells were harvested and the metaphase plates examined. Cells were scored as being from the original

thymus graft donor (T6T6), the thymus graft recipient (Lac) or the added peripheral blood cell population (Lac x T6T6). The results shown in Figure 8 do not include those F_1 (Lac x T6T6) peripheral blood cells responding but just show the percentage of cells with the "persistent" (T6T6) karyotype compared with the "non-persistent" karyotype (Lac). It can be seen that in the earlier fractions collected there was a high percentage of "persistent" cells responding, but by fraction 15 no cells showing the "persistent" karyotype could be seen, and all the cells seen in this fraction had the karyotype of the thymus graft recipient (Lac). Although there was only slight enrichment of "persistent" cells in fractions 5 and 6, there was a considerable diminution in the percentage of these cells responding in the later fractions (12-15). From this experiment it could be seen that the "persistent" population could be separated from at least some cells of the "non-persistent" population.

A further experiment was performed, which differed from the one just described in that the animals bearing thymus grafts were treated with hydrocortisone three days prior to graft removal. In this experiment 200×10^6 hydrocortisone-treated thymus graft cells (showing a viability of 80%) taken from CBA/Lac mice that were grafted subcutaneously 82 days previously with

18 syngeneic but chromosomally marked newborn thymuses (CBA/H.T6T6), were placed on the gradient and sedimented for 2 hours at room temperature. One million fractionated thymus graft cells were mixed with one million (Lac x T6T6) F_1 peripheral blood lymphocytes and cultured with PHA for 3 days. In this experiment cell recovery was only 33% of those cells placed on the gradient, and viability ranged from 96% in fraction 12 down to only 47% in fraction 19. In all cases the viability of the cells from fractions 15-20 was less than the 80% viability of the starting cell population. This could be due either to the fact that dead cells do not sediment well in this system or that the non-viable cells were part of the slow sedimenting population. The latter seems the most likely explanation since it is known that after hydrocortisone treatment the thymus cortex contains many dead or dying cells and it is thought that the cortical cell population is the slow sedimenting population in this system.

After 3 days in culture (colcemid being added during the last 5 hours of culture) the cells were harvested and the metaphase plates examined. Cells were scored as being from the original thymus graft donor (T6T6), the thymus graft recipient (Lac) or the added peripheral blood cell population (Lac x T6T6). The results shown in Figure 9 do not include those F_1 (Lac x T6T6)

peripheral blood cells responding but just show the percentage of cells with the "persistent" (T6T6) karyotype compared with the "non-persistent" karyotype (Lac). It can be seen that in the earlier fractions collected there was a high percentage of "persistent" cells responding, but by fraction 20 no "persistent" cells could be seen. There was some enrichment of "persistent" cells in the first four fractions, but after fraction 15 there was a considerable drop in the percentage of these cells responding.

From this and the previous experiment it would seem that the PHA-responsive population bearing the "persistent" karyotype could be separated physically to some extent from that population bearing the "non-persistent" karyotype. This separation is not complete since even in the lowest fractions examined, cells bearing the "non-persistent" karyotype could be seen. This observation could be interpreted as showing that some cells with the "non-persistent" karyotype were in fact cells that persist in the thymus and have all the characteristics of the demonstrable "persistent" population, whereas other "non-persistent" cells have in fact different sedimentation properties in this system and were found nearer to the top of the gradient. If Miller and Phillips (1969) are right in their notion that in this system larger cells sediment faster than smaller cells, then it would seem that

the "persistent" population would consist of larger cells than the "non-persistent" population. When certain fractions were examined under the microscope for cell size it could be seen that in general cells from the lower fractions were larger than those from the higher fractions although no quantitative evaluation of size was made.

One troublesome feature of these experiments was the fact that only a certain percentage of the cells put on the gradients could be recovered at the end. In the three experiments reported recovery was only 63%, 51% and 33% respectively of the initial cell populations. With these figures in mind it can be seen that only selected cell populations have been evaluated for their PHA-responsive cells. With this reservation in mind it is concluded that it is possible to some extent to separate "persistent" from "non-persistent" PHA-responsive cells on these gradients and that therefore we are dealing with true heterogeneity among the PHA-responsive cell population in the thymus.

Figure 7

Tritiated thymidine incorporation by PHA-stimulated mouse thymus graft cells separated on a 0.2% to 2% BSA gradient.

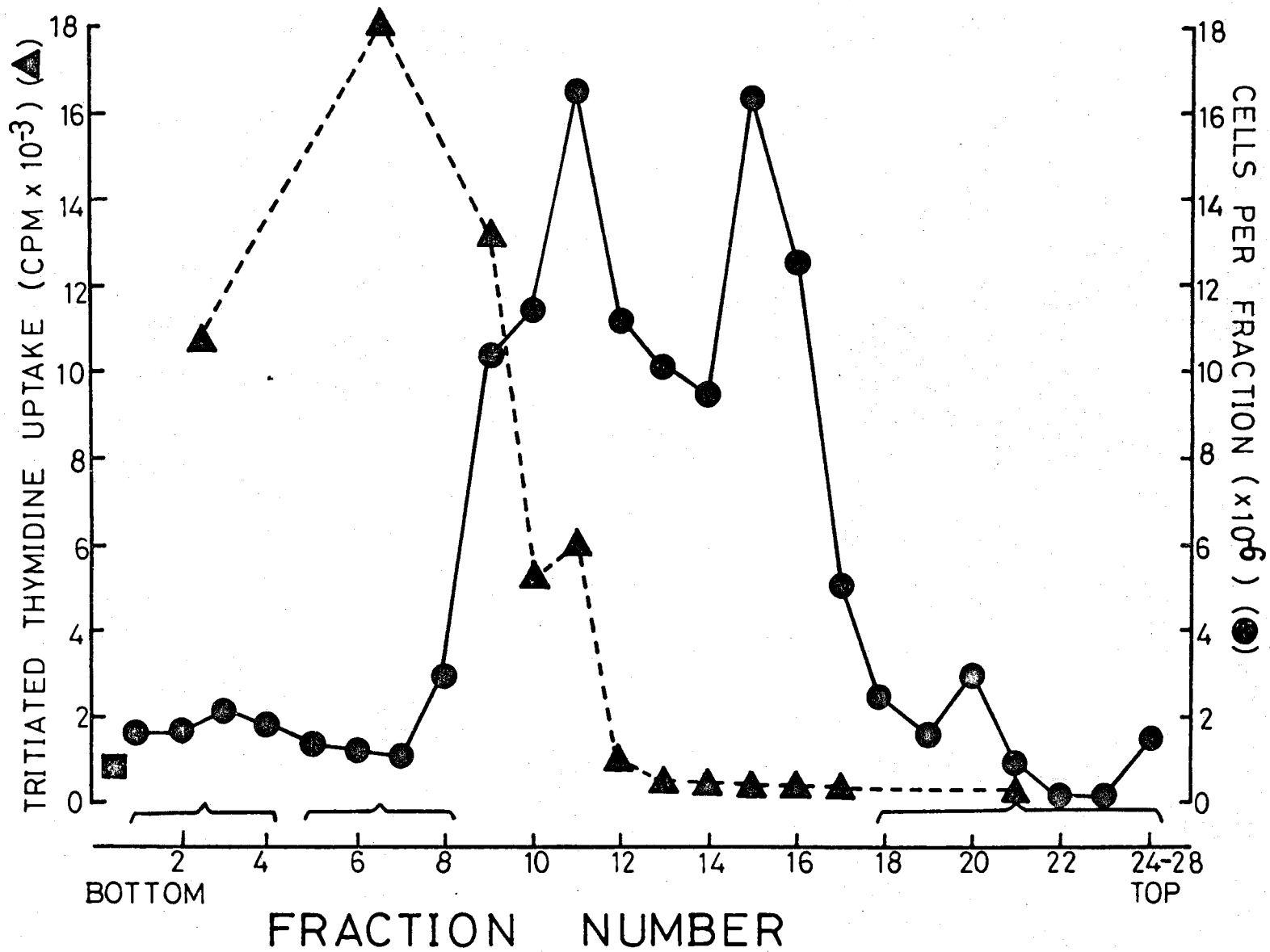


Figure 8

The percentage of persistent PHA-responsive cells from mouse thymus graft cells separated on a 0.2% to 2% BSA gradient.

An average of 56 mitotic figures of thymus graft origin were counted in each fraction.

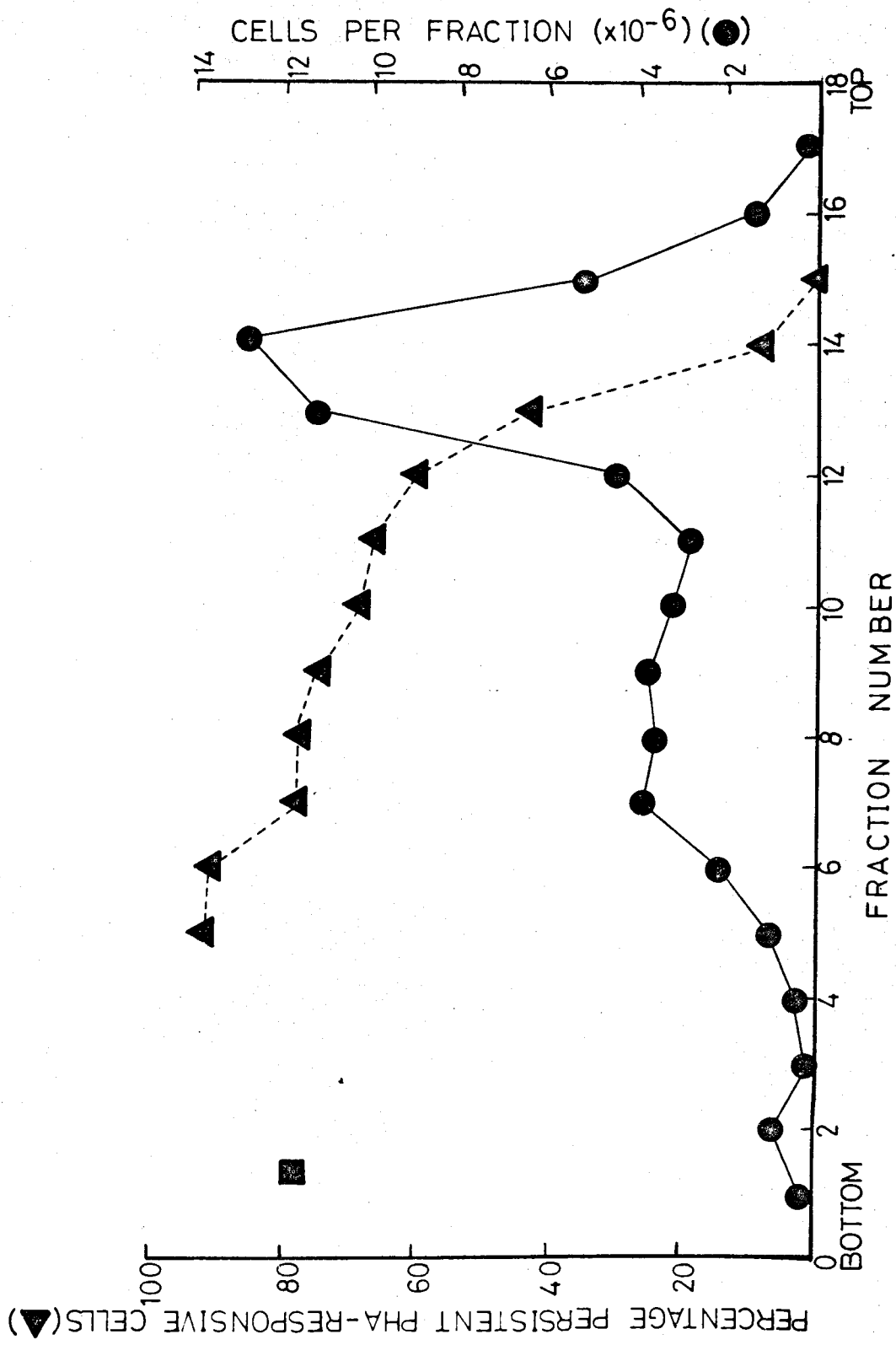
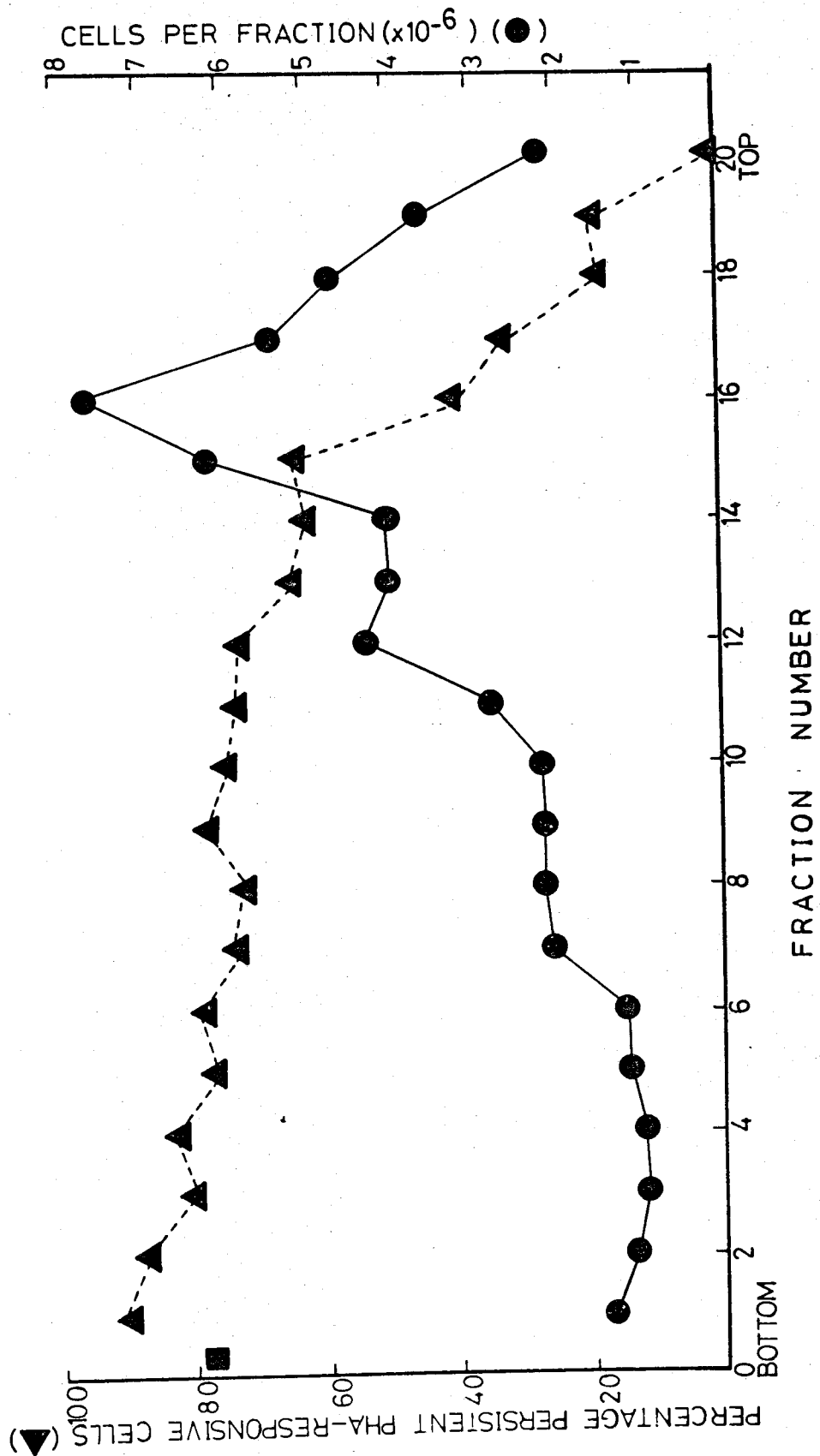


Figure 9

The percentage of persistent PHA-responsive cells from hydrocortisone-treated mouse thymus graft cells separated on a 0.2% to 2% BSA gradient.

An average of 35 mitotic figures of thymus graft origin were counted in each fraction.



BONE MARROW ORIGIN OF PERSISTENT
PHA-RESPONSIVE CELLS IN MOUSE THYMUS GRAFTS

Although in mouse embryogenesis it seems likely that stem cells responsible for thymic lymphopoiesis are derived from yolk sac and foetal liver (reviewed by Owen, 1972), there is now ample evidence that thymus grafts and irradiated thymuses in situ can be repopulated by cells of bone marrow origin (Dukor et al., 1965; Micklem and Loutit, 1966). With the knowledge of the existence of a persistent PHA-responsive cell population in the mouse thymus it was of interest to determine where these cells originate. The basic question to be answered was whether the stem cells responsible for the production of the persistent population originated in situ, for instance from epithelial elements in the thymus, or from an imigrant blood borne stem cell population probably of bone marrow origin. Since at the time of thymus grafting stem cells responsible for the production of the persistent population must already have been present in the thymus graft, a considerable problem concerning their origin was presented.

In undertaking this problem one basic assumption was made. This was that a majority of the PHA-responsive cells were

cells that persisted in syngeneic thymus grafts even when the persistent karyotype could not be demonstrated. With this assumption in mind various experimental manipulations were performed to determine the origin of the stem cells responsible for the persistent PHA-responsive cell population.

(a) Irradiated Newborn Thymuses

In 1965, Dukor and his coworkers showed that the mitotic cells in subcutaneous mouse thymus grafts were initially of graft type but that between 15 and 22 days after grafting there was a changeover in the type of cell in division, so that by 22 days post grafting all dividing cells were of host type. In another experiment they grafted animals subcutaneously with newborn thymuses which had been irradiated in vitro with 2000R. When they sampled these grafts for mitotic figures they could find none of thymus graft donor type at any time studied even when donor-type mitoses could be seen in the control unirradiated thymus grafts. Their explanation of this finding was that the stem cells in the unirradiated newborn thymus, which could proliferate albeit only for 15 days or so in syngeneic hosts, were destroyed by 2000R.

With these results in mind, an experiment was performed to determine if the PHA-responsive population in irradiated grafts

would be of host or donor type. In the first experiment newborn CBA/H.T6T6 thymuses were given 2000R in vitro, and then transplanted under the kidney capsule of CBA/Lac mice. Fifty six days later the animals were killed and their kidneys were examined for the presence of thymus grafts. Unfortunately in none of the animals examined could a thymus graft be found.

In the next experiment the dose of irradiation received in vitro by the newborn thymuses was dropped from 2000R to 667R. These CBA/H.T6T6 thymuses given 667R in vitro, were grafted under the kidney capsules of CBA/Lac mice, as were control thymuses that were sham-irradiated. The animals were killed at either 41 or 117 days after grafting and the thymus grafts were removed and teased, and the cells washed twice in medium 199 and counted. Approximately one million thymus graft cells were mixed with one million (Lac x T6T6) F_1 peripheral blood lymphocytes and cultured with PHA for 3 days. After harvesting, metaphase plates were scored as being from the thymus-graft donor (T6T6), the thymus graft recipient (Lac) or the added (Lac x T6T6) F_1 peripheral blood lymphocytes. Figure 10 is a plot of the percentage of donor (persistent)-type PHA-responsive cells responding (F_1 mitotic figures excluded), against the number of cells found in the same thymus grafts after washing

twice in medium 199. It can be seen that irradiated grafts showed a much lower percentage of donor-type PHA-responsive cells than did unirradiated grafts at both times studied.

Unfortunately, along with this lower percentage of donor-type PHA-responsive cells, these irradiated thymus grafts contained considerably fewer cells in them than did control unirradiated thymus grafts, and could not therefore be considered as anywhere near normal. Even though these grafts received 667R there was still an appreciable percentage of PHA-responsive cells of donor-type as late as 117 days post irradiation, and from this it can be concluded that this amount of irradiation did not completely destroy the stem cells responsible for the production of the persistent PHA-responsive cells. From these experiments no conclusion was drawn as to the origin of the persistent cell population.

(b) Squashed Newborn Thymuses

With the knowledge that the stem cells responsible for the production of the persistent PHA-responsive cell population were in the newborn thymus at the time of grafting, another approach was sought to eliminate them. Once again the assumption was made that after elimination the thymus would still produce a persistent PHA-responsive cell population even

though this population might not show the persistent karyotype.

In the experiment to be described, the method used to try to eliminate these stem cells from the newborn thymus was by squashing the thymuses and expelling as many cells as possible from them before transplantation under the kidney capsules of recipient mice. This was achieved by placing an individual newborn thymus lobe on a glass slide. The ends of the thymus were cut off with a scalpel blade and the cells squashed from the thymus by placing on top of the thymus another glass slide and exerting gentle pressure. Many cells were in this way expressed from the thymus, and the remnant was grafted under the kidney capsule of a recipient mouse. The control in this experiment was thymuses which just had their ends cut off but were not squashed. The squashed newborn thymuses were of CBA/H.T6T6 origin and the recipients were CBA/Lac mice. 44 days after grafting the thymus grafts were removed and teased, and the resulting cell suspensions were washed twice in medium 199 and counted. One million thymus graft cells were added to one million (T6T6 x Lac) F_1 peripheral blood lymphocytes and cultured with PHA for three days. After harvesting, the metaphase plates were scored as being from the thymus graft donor (T6T6), the thymus graft recipient (Lac) or

the added F_1 peripheral blood lymphocytes. Figure 11 is a plot of the percentage of PHA-responsive cells with the "persistent" karyotype (T6T6) (F_1 cells excluded) against the number of cells in the thymus graft after two washes. It can be seen that thymuses which just had their ends cut off gave a high percentage of cells with the "persistent" karyotype, whereas those that were squashed in addition, showed variable percentages of cells with the "persistent" karyotype. In addition it can be seen that both types of treatment gave thymus grafts containing almost the same number of cells (\bar{x} 8.5×10^6 squashed v \bar{x} 8.4×10^6 cut ends).

In conclusion it seems that removing most of the cells from the newborn thymus at the time of grating, did result usually in a lowering of the percentage of PHA-responsive cells with the "persistent" karyotype. The results however were very variable and therefore not conclusive, but did suggest, if the basic assumption made was correct, that there are very few stem cells in a newborn thymus that can give rise to the persistent population, and that these cells are extremely difficult to remove from the grafts by such a simple procedure as squashing.

(c) Newborn Thymuses in Millipore Chambers

There is now ample evidence that thymuses enclosed in cell impermeable millipore chambers can restore, albeit in a

small way, immunological responsiveness to certain immunologically crippled mice. Examination of such thymuses suggests that this reconstitution is brought about by products of thymic epithelial cells, since, soon after grafting these thymuses show few signs of lymphocytes or lymphopoiesis (reviewed by Miller and Osoba, 1967). With these facts in mind it was considered likely that the stem cells responsible for the production of the persistent PHA-responsive cell population might be eliminated if the newborn thymuses were first cultured in millipore chambers in a suitable recipient animal before being transplanted under the kidney capsule of the final recipient.

Again using the assumption that a majority of the PHA-responsive cells were cells that persisted in syngeneic thymus grafts even when the "persistent" karyotype could not be demonstrated, experiments were undertaken to ascertain whether these stem cells were derived from epithelial elements in the thymus or from immigrant stem cells, possibly bone marrow in origin.

Thymuses from newborn CBA/Lac mice were placed in cell impermeable millipore chambers (0.2 micron porosity) and the chambers were sealed with acetone. These chambers were placed into the peritoneal cavities of normal CBA/H.T6T6 recipient mice. After either 5 or 11 days the mice were killed

and the chambers were removed. The thymus graft remnants were removed from the chambers and grafted under the kidney capsules of further normal CBA/H.T6T6 recipient mice. Fifty six days later these mice were killed and the thymus grafts removed and teased. The resulting cell suspensions were washed twice in medium 199 and counted. Thymus graft cells were cultured with (Lac x T6T6) F_1 peripheral blood lymphocytes and PHA for 3 days. After harvesting, the metaphase plates were scored as being from the original thymus graft (Lac), the thymus graft recipients (T6T6), or the added F_1 peripheral blood lymphocytes.

Figure 12 is a plot of the percentage of PHA-responsive cells with the "persistent" karyotype (Lac) (F_1 cells excluded) against the number of cells in the thymus graft after two washes. It can be seen that when grafts were allowed to remain for only 5 days in millipore chambers, two out of three of the grafts had a high percentage of PHA-responsive cells showing the "persistent" karyotype, whereas if left for 11 days three out of four grafts showed no such cells at all, and the fourth had only a very low percentage of cells with the "persistent" karyotype. The number of cells found in these thymus grafts was variable, but did not seem to correlate with the percentage of cells bearing the

"persistent" karyotype.

The conclusion drawn from this experiment, again assuming that the basic assumption is right, is that the stem cells responsible for the production of the "persistent" cell population are derived from a source outside the thymus (probably bone marrow in origin) and not from elements of the thymic epithelial framework.

(d) Thymuses in situ

In 1966, Micklem and his coworkers showed that mice that were subjected to potentially lethal body irradiation (1000R) and injected with syngeneic bone marrow cells, could regenerate the mitotic population of cells within their own thymus, and that during the initial stages at least, the cells in division were of host type and not derived from the bone marrow cell inoculum. This meant that the stem cells responsible for this host-derived mitotic population had survived 1000R irradiation, at least long enough to produce a substantial number of mitotic lymphocytes as late as 14 days post irradiation. Armed with this knowledge it was considered a possibility that in fact some host-derived cells might differentiate further in the thymus and eventually enter the PHA-responsive cell population. If this was the case, could some of these PHA-responsive cells persist in the thymus for any

length of time. Preliminary experiments showed that with the standard bone marrow cell inoculum (5×10^6), few cells ($\approx 20\%$) could be demonstrated to be responding to PHA and have the host (i.e. irradiated) karyotype. From a number of so far unpublished experiments carried out in Dr Davies' laboratory, it seemed that if the number of bone marrow cells injected was reduced, then the onset of bone marrow derived divisions in the thymus was delayed. With these results in mind, experiments were undertaken to determine if lowering the number of bone marrow cells injected allowed time for the host derived stem cells in these thymuses to produce higher percentages of host-derived PHA-responsive cells.

Adult CBA/Lac mice were irradiated (850R) and injected intravenously within 3 hours of irradiation with various numbers of CBA/H.T6T6 bone marrow cells (10^5 to 10^7). At either 47 or 110 days after irradiation the animals were killed and their thymuses were removed and teased. After two washes in medium 199 the cells were counted and placed in culture with (Lac x T6T6) F_1 peripheral blood lymphocytes and PHA for 3 days. After harvesting the metaphase plates were scored for cells of host origin (Lac), bone marrow origin (T6T6), or F_1 peripheral blood lymphocyte origin.

Table 11 shows the number of nucleated cells obtained from these thymuses (after two washes). It can be seen that there were only about one third the number of cells at 110 days as there were at 47 days. This could be explained by the fact that with age, the thymus is known to involute (reviewed by Dougherty, 1952). One striking feature of these results was that there appeared to be no significant differences, whatever number of bone marrow cells were injected. Thus the thymus was able to regenerate to the same cellularity with 10^5 right up to 10^7 bone marrow cells injected.

When the PHA-responsive cell population was examined 47 days after irradiation, it could be seen (Figure 13) that when small numbers of bone marrow cells were injected there was a high percentage of host cells responding. As the number of bone marrow cells injected increased, so the percentage of host-derived PHA-responsive cells declined. When thymuses were examined at 110 days post irradiation for the presence of host-type PHA-responsive cells, the percentage, that was high at 47 days in the groups given lower numbers of bone marrow cells, was reduced, so that in all groups it ranged between 14 and 24 percent.

There has been some difficulty in interpreting these

results. It would seem that injection of small numbers of bone marrow cells allowed the proliferation of host-derived stem cells in the thymus to a far greater extent than did the injection of large numbers of bone marrow cells. We already know that bone marrow cells can repopulate the thymus, and as mentioned above, preliminary results from this laboratory indicate that repopulation is accomplished faster with large numbers of bone marrow cells. It would thus seem in the 47 day experiment described above, the irradiated host cells responsible for the production of host-derived PHA-responsive cells are given sufficient time to proliferate in the presence of small numbers of bone marrow cells whereas with large numbers of bone marrow cells there may be competition for space in the thymus, a contest which the bone marrow cells eventually win.

The 110 day results are perplexing, but would seem to suggest that the PHA-responsive cells seen at day 47 with the host karyotype, are relatively short lived. Since we know that the persistent population of PHA-responsive cells found in normal thymus grafts is long lived, there are two possibilities to consider. We know that the host-derived PHA-responsive population derives from stem cells that received 850R irradiation, so that either the host-derived PHA-responsive cell has a limited capacity of survival

in the thymus due to the effects of irradiation of its stem cells, or these cells are continually being replaced by host-derived stem cells that have survived irradiation but which have a limited life span due to the effects of irradiation. The former possibility seems the most likely since no cells showing the persistent karyotype could be shown proliferating in thymus grafts later than 25 days post grafting (Leuchars et al., 1967), or in radiation chimaeras as late as 100 days post irradiation (Koller et al., 1966). Just to confirm that no graft-type cells were proliferating at late times after grafting an experiment was performed. CBA/Lac mice were grafted under the kidney capsule with one lobe of a newborn CBA/H.T6T6 thymus. Fifty days later the animals were injected with colcemid, and one hour later the thymus grafts were removed, teased and prepared for cytological examination. In the 4 mice examined 400 cells were scored, all of which showed the thymus graft recipient (Lac) karyotype. Thus no cells of thymus graft type could be seen dividing in the grafts at 50 days post grafting.

Once again, using the basic assumption that a large majority of PHA-responsive cells in the thymus are persistent even though they might not show the "persistent" karyotype, it can be concluded that in these experiments where few cells

responding to PHA had the "persistent" karyotype, many cells did persist and in fact had the karyotype of the injected bone marrow cell population.

(e) Conclusions

Nearly all of the experiments reported above suggest that the persistent PHA-responsive cell population did in fact derive from stem cells that initially originate outside the thymus, and not from stem cells derived from the thymus itself. Other circumstantial evidence is also suggestive of this notion. We know that peripheral T cells are θ -positive (reviewed by Raff, 1971b) and derived from stem cells that enter the thymus via the blood stream from the yolk sac and foetal liver in early life, and probably from the bone marrow in adult life (reviewed by Owen, 1972). In a previous section it was shown that the persistent PHA-responsive cell population was θ -positive, and it seems likely that the peripheral T-cell and the "persistent" and "non-persistent" PHA-responsive cells found in the thymus have a common stem cell, which gives rise to cell populations which either leave the thymus to become peripheral T-cells or stay in the thymus to give the persistent PHA-responsive cell population.

One cautionary note should be sounded. Although the evidence presented above is suggestive of a bone-marrow origin

for persistent PHA-responsive cells, it is not proof. Until another marker for the persistent state is discovered, caution in interpretation of these results is advisable.

Figure 10

The percentage of persistent PHA-responsive cells and the number of cells in thymus grafts that had been irradiated (667R) in vitro before implantation. An average of 31 mitotic figures of thymus graft origin were counted.

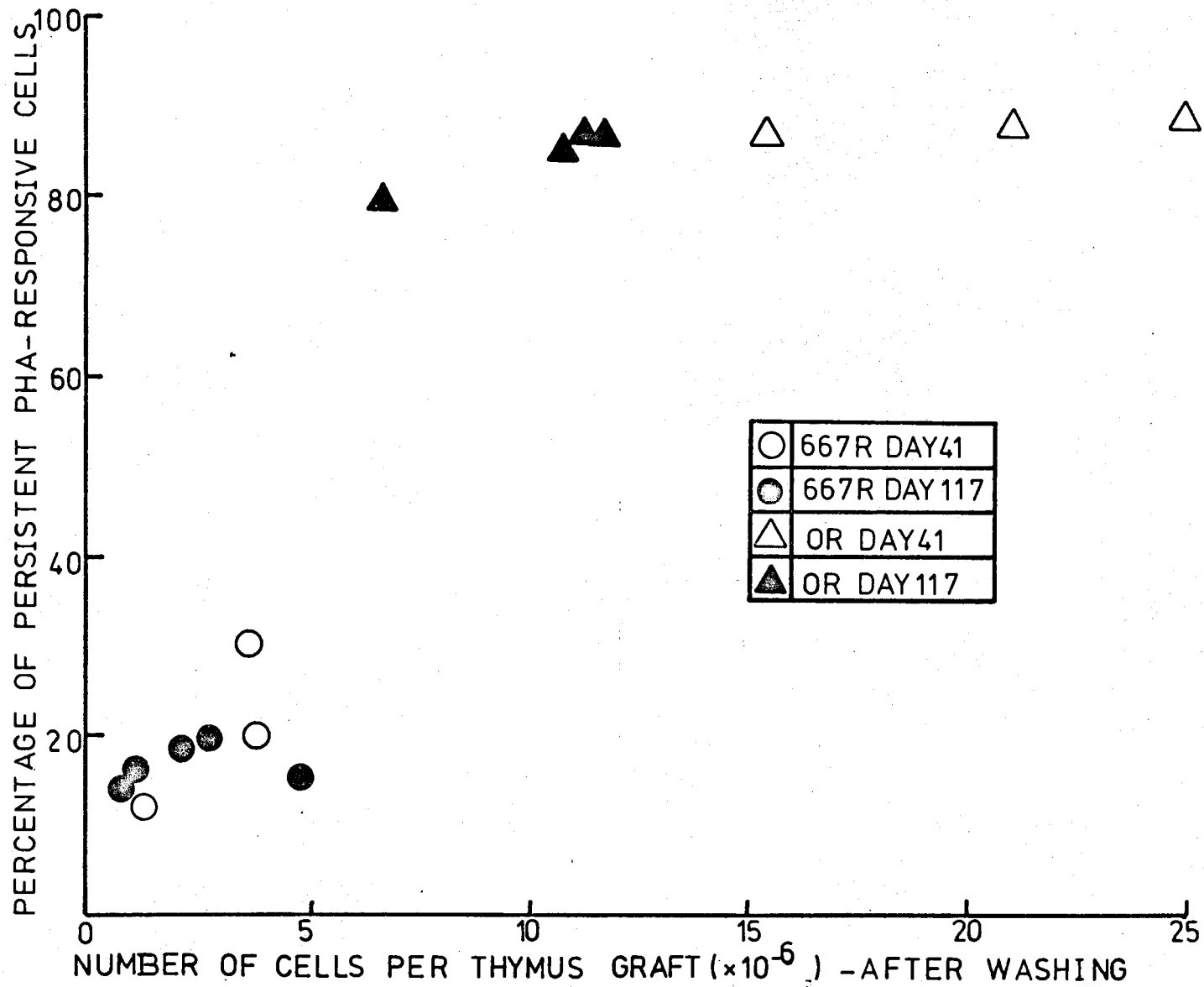


Figure 11

The percentage of persistent PHA-responsive cells and the number of cells in thymus grafts, that on the day of grafting were either squashed or not squashed. An average of 39 mitotic figures of thymus graft origin were counted.

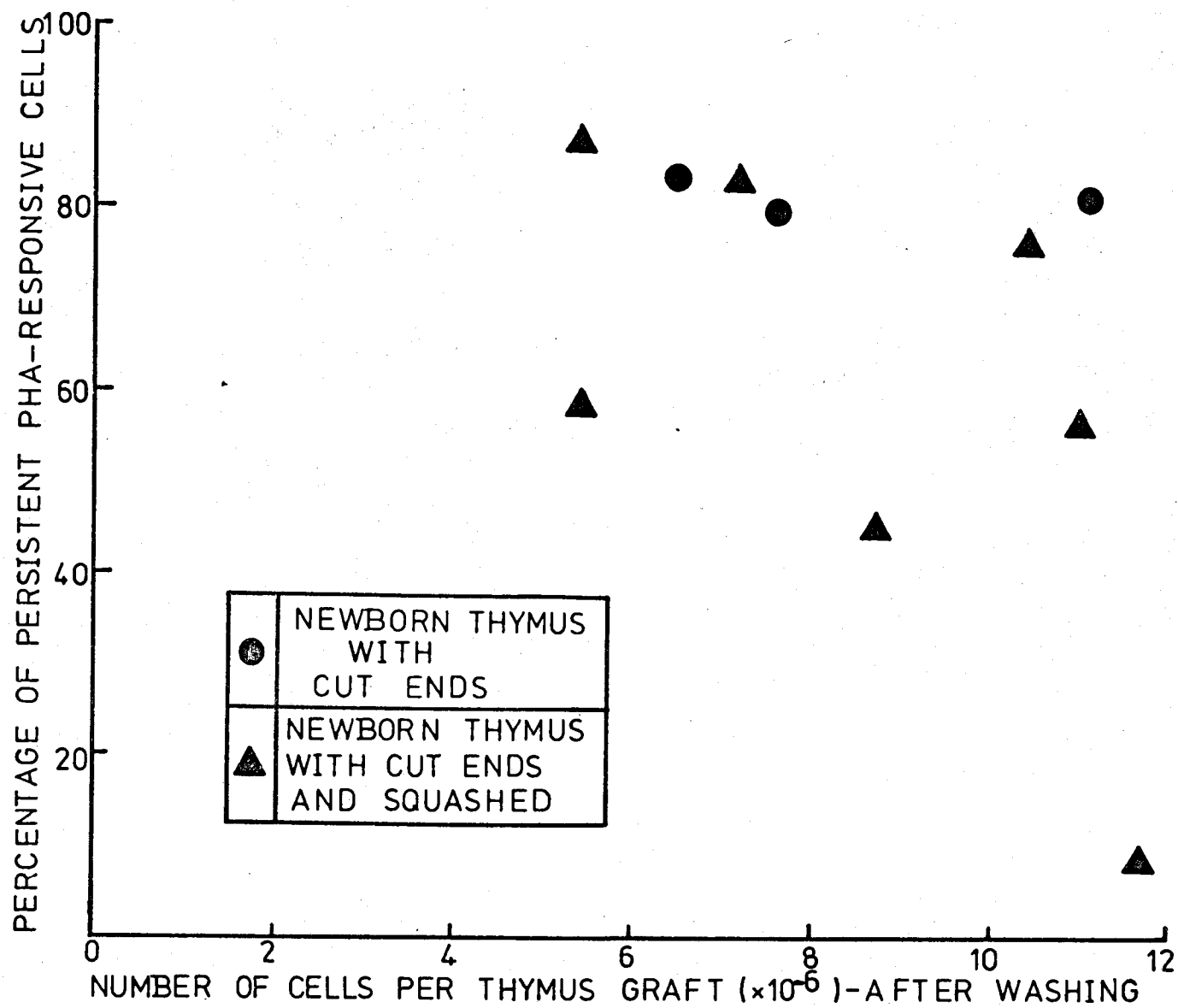


Figure 12

The percentage of persistent PHA-responsive cells and the number of cells, in thymus grafts that had been passaged in vivo in millipore chambers. An average of 23 mitotic figures of thymus graft origin were counted.

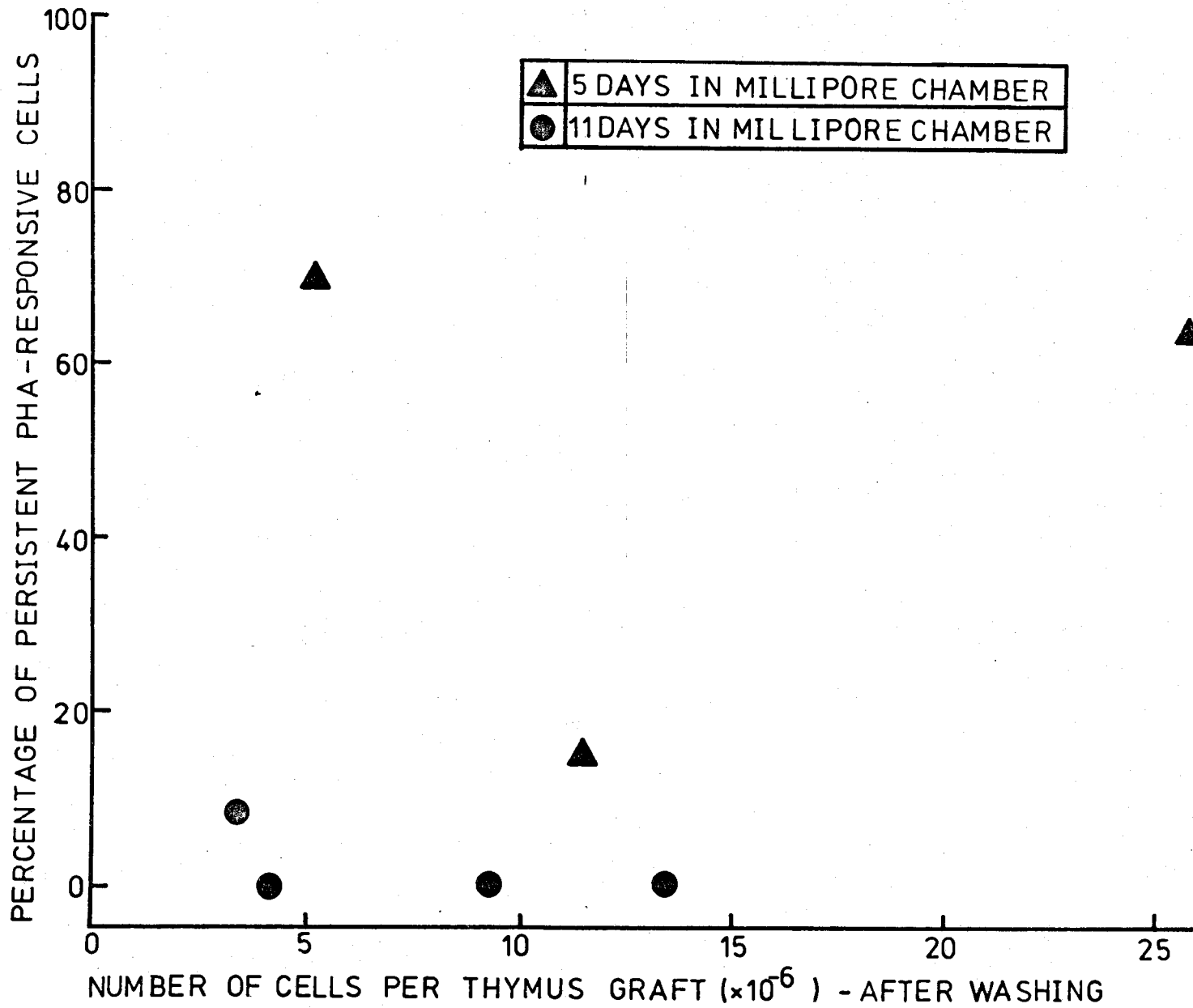


TABLE 11

Number of cells per thymus ($\times 10^{-6}$) of radiation chimaeras at 47 and 110 days after irradiation and bone marrow inoculation.

Number of Bone Marrow Cells Injected	Day 47	Day 110
1×10^5	66.0 *	20.8
5×10^5	58.0	18.8
1×10^6	64.6	15.6
5×10^6	52.2	20.8
1×10^7	54.6	14.4

* 3 mice per group

Figure 13

The percentage of PHA-responsive cells of host type in the thymuses of radiation chimaeras 47 days after irradiation (850R) and bone marrow therapy. The bars represent \pm two standard errors from the mean (3 mice per group), and the line is drawn by eye. An average of 91 mitotic figures of thymus graft origin were scored per group.

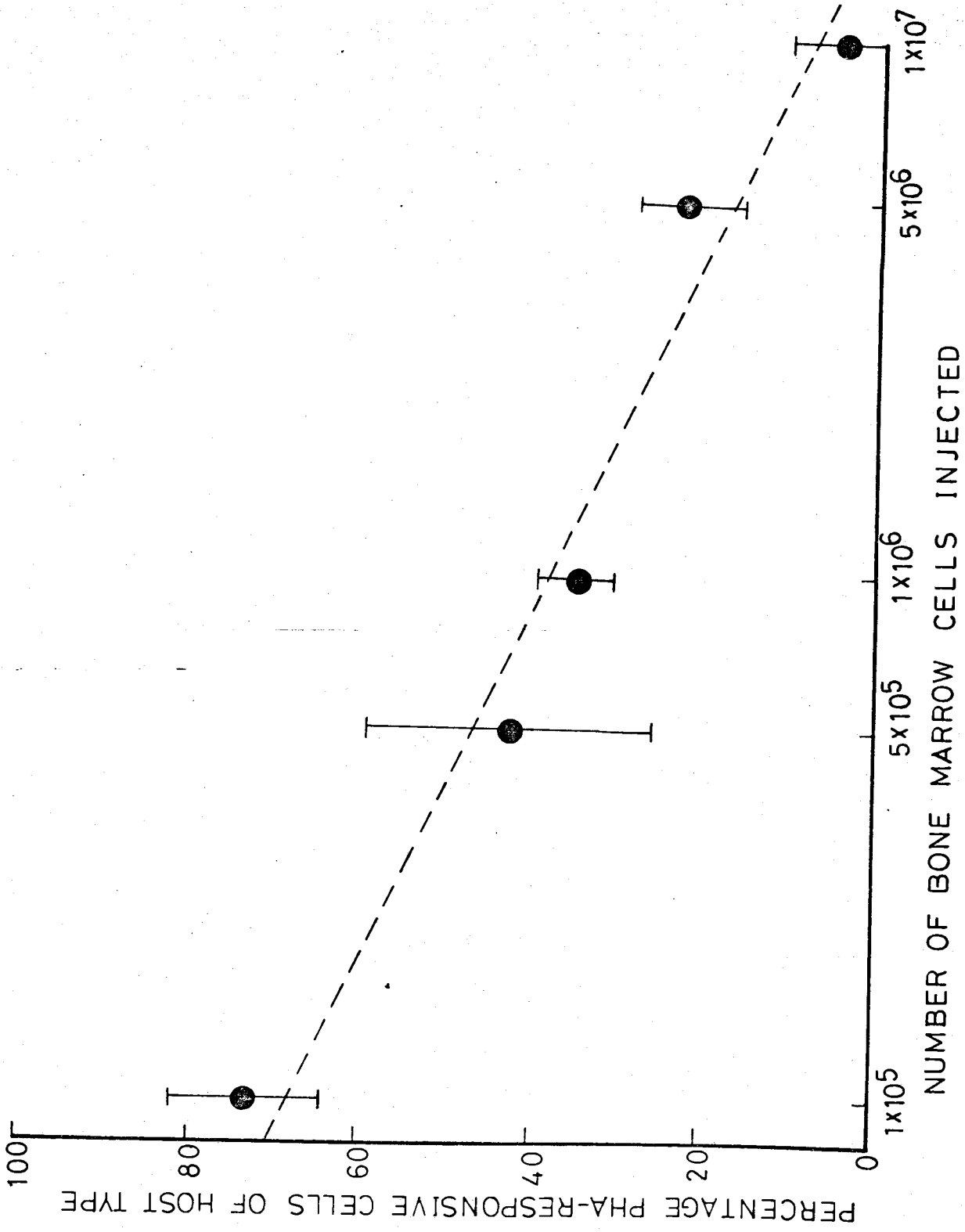
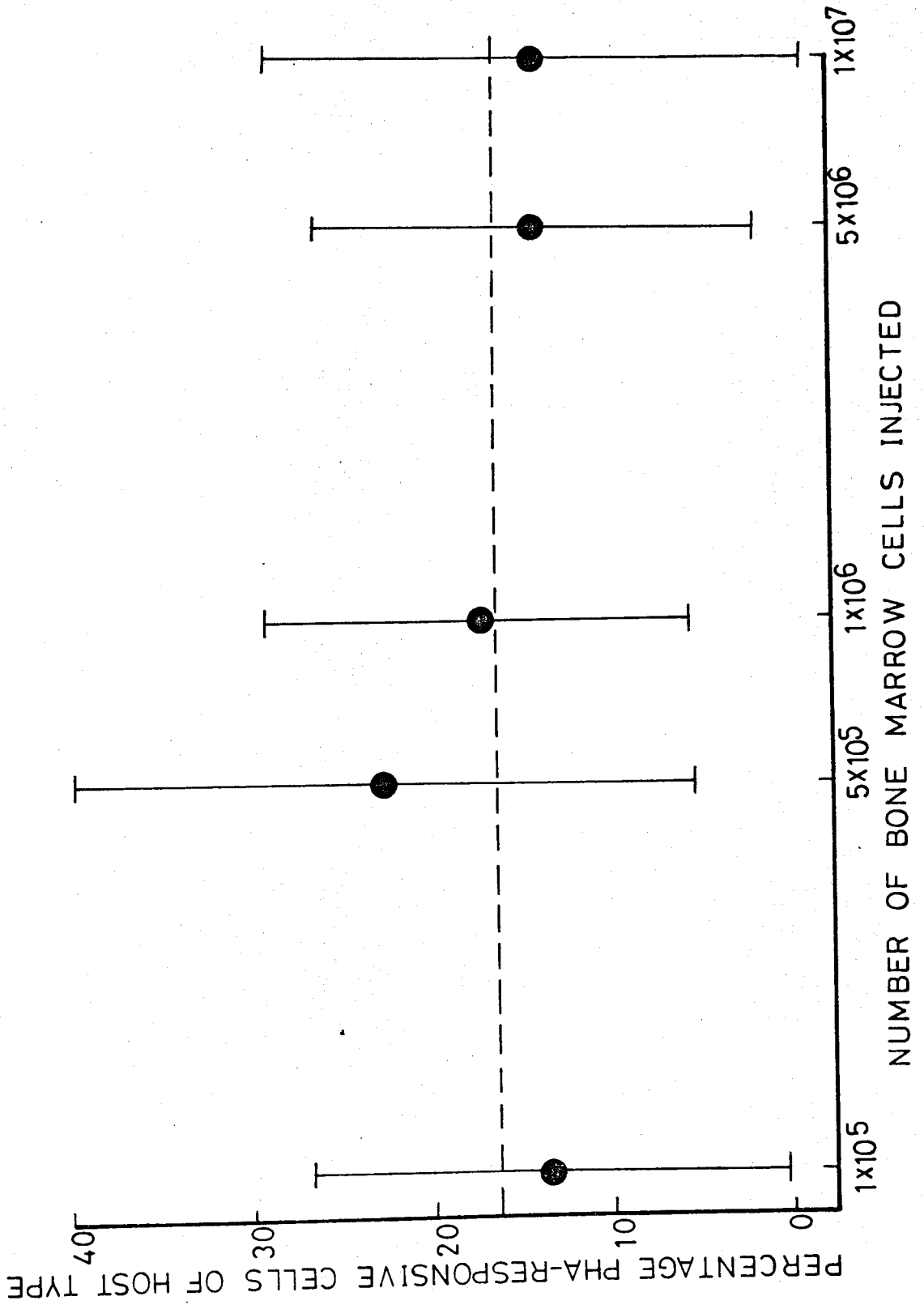


Figure 14

The percentage of PHA-responsive cells of host type in the thymuses of radiation chimaeras 110 days after irradiation (850R) and bone marrow therapy. The bars represent \pm two standard errors from the mean (3 mice per group), and the line is drawn by eye. An average of 82 mitotic figures of thymus graft origin were scored per group.



RECIRCULATORY CHARACTERISTICS OF PERSISTENT PHA-
RESPONSIVE CELLS FROM MOUSE THYMUS GRAFTS

There is now overwhelming evidence showing that some cells at least, migrate from the thymus to peripheral lymphoid tissues, where they are known as T-lymphocytes (Miller, 1962; Weissman, 1967; Davies, 1969). More recently, experiments have shown that thymocytes can be made to migrate to the spleen and lymph nodes of recipient mice (Lance et al., 1971; Raff, 1971). Since it has been shown in previous sections of this thesis that a majority of PHA-responsive cells persist in the thymus for long periods, it was of interest to see if these cells could home to lymphoid organs in suitable recipient mice.

Table 12 and 14 show the experimental protocols of two such experiments. Basically they are very similar and differ only in small details. In both experiments normal CBA/Lac mice were grafted subcutaneously with 14-18 single thymus lobes from newborn (Lac x T6T6) F_1 mice, and either 40 or 70 days later the grafts were removed and teased. After several washes in medium 199 the cells were counted and some were placed in culture with CBA/H.T6T6 peripheral blood cells and PHA. The rest of the cells were injected intravenously into thymectomized radiation chimaeras (Tx T6T6 given 850R and

5×10^6 T6T6 bone marrow cells). In the first experiment (Fig. 12) 100×10^6 thymocytes were injected into chimaeras that had been irradiated 30 days previously, while in the second experiment (Fig. 14) 30×10^6 thymocytes were injected into chimaeras that were irradiated and bone marrow injected on the same day as thymocyte injection. In Table 12, half the animals were painted with oxazolone 97 days after thymocyte injection, and three days later the lymph nodes were prepared for cytological analysis. The spleen and peripheral blood lymphocytes of these same animals were cultured with PHA for 3 days. The other half of the mice were killed 100 days after thymocyte injection, and their lymph node, spleen and peripheral blood lymphocytes were cultured with PHA for 3 days. In Table 14, half the animals were painted with oxazolone 81 days after thymocyte injection, and three days later the lymph nodes and spleen were prepared for cytological analysis. The peripheral blood lymphocytes of these same animals were cultured with PHA for 3 days. The other half of the mice were killed 84 days after thymocyte injection, and their lymph node, spleen and peripheral blood lymphocytes were cultured with PHA for 3 days. The organs and cultures were prepared for cytological examination as previously described, and cells were scored as being from the original thymus graft

donor (Lac x T6T6), the original thymus graft recipient (Lac) or the thymectomized radiation chimaera (T6T6). In the results presented in Tables 13 and 15, the mitoses derived from the radiation chimaera (T6T6) have been excluded and the results are expressed as a percentage of cells of thymus graft origin responding.

It can be seen in Table 13 A that over 70% of the cells responding to PHA in the original thymus graft cell suspension were of (Lac x T6T6) F_1 origin. That is to say they had persisted in the thymus graft and could be found 70 days after grafting. When the 3 day oxazolone response (a 100% T-cell response at this time - Kerbel, Elliott and Wallis, 1974) of lymph nodes was examined (Table 13 B), it could be seen that very few mitotic cells of the original thymus graft donor type (F_1) could be found. The same was true for F_1 cells in the spleens of oxazolone-treated mice when the cells were cultured with PHA. The only place where an appreciable percentage of F_1 thymus graft cells could be seen responding to PHA was in the peripheral blood. Here, nearly 40% of the cells responding were of F_1 type, still far lower than the 70% found responding in the original thymocyte suspension. In animals that were not painted with oxazolone much the same results were obtained (Table 13 C).

In the spleens and lymph nodes of these animals there were very few cells of F_1 type that could respond to PHA, although the figures were higher than those of oxazolone-treated animals. Once again appreciable numbers of F_1 cells could be found in peripheral blood (nearly 32%) although still less than the percentage seen in the initial starting cell population. These results were interesting, and the experiment was repeated with some minor alterations as previously described. One of these alterations was that instead of taking the spleens from oxazolone stimulated animals and culturing with PHA, they were examined cytologically straight from the animal after the appropriate injection of colcemid. This alteration was made after it was shown that cutaneous sensitization of mice with oxazolone did stimulate cell proliferation in the spleen as well as in the draining lymph nodes (Micklem, Ogden and Pritchard, 1972). The results, considering the alterations in experimental procedure, were remarkably similar. Although nearly 80% of the cells responding to PHA in the original cell suspension were of F_1 type, only very few F_1 cells could be seen responding in the spleen and lymph nodes of oxazolone-treated mice. In the spleen and lymph nodes of non-oxazolone-treated mice there were still few cells of F_1 type responding to PHA. In both experiments there

was a higher percentage of cells of F_1 type in the spleens and lymph nodes of non-oxazolone-treated mice than in the corresponding mice painted with oxazolone. In both experiments, however, these differences were not significant when analysed by the students "t" test ($p > 0.05$). In both groups (Table 15 B and C), the peripheral blood showed a higher percentage of F_1 cells than did spleen or lymph node ($p < 0.01$).

A number of conclusions can be drawn from these experiments. Firstly, it would appear that the persistent PHA responsive population (F_1 in this case) does not localize very well to spleen or lymph nodes. There is no evidence presented here on the initial localization of these cells, but by 84 or 100 days post injection, very few of them can be found in the spleen or lymph nodes. Secondly, an even smaller percentage of the cells responding to the skin sensitizing agent oxazolone were of F_1 origin, and although the results were not significant, they do suggest that the persistent cell population may not respond well to antigen. Thirdly, although there was a diminution in the percentage of "persistent" cells in the peripheral blood of recipient animals when compared to their responsiveness in the initial population, there was still an appreciable percentage of them at 84 and 100 days after transfer. The most likely

explanation of this drop in percentage is that the "persistent" cell population has a limited life span outside the thymus, and that with time the percentage decreases (cf. \bar{x} 45% at 84 days and \bar{x} 35.4% at 100 days). Attempts were made to sample the peripheral blood of these animals on a number of occasions prior to killing, but the PHA-stimulated cultures did not grow. Finally, it would seem that the persistent PHA-responsive population, when injected into an appropriate recipient, can circulate in the peripheral blood of that recipient without becoming extravascularized in the spleen or lymph nodes. It would thus seem to have the properties of circulation in the blood but not of recirculation from blood-lymph-blood. One proviso should be made to this conclusion, which is that this cell population may indeed be found in the spleen and lymph nodes, but not in a condition whereby it can respond to PHA or oxazolone.

It appears from the preceding experiments that the persistent PHA-responsive cell population has an unusual distribution when injected into deprived recipients. These cells would not seem to recirculate from blood-lymph-blood, but appear to be trapped in the circulation for long periods. One possibility to be considered is that this cell population can only be extravascularized in the thymus but not in other lymphoid

organs. Unfortunately, this hypothesis was not tested for directly, that is by seeing if these cells in the peripheral blood could home to thymus. Another experiment was however performed, that tested whether the persistent cells in a thymus could leave the thymus, circulate, and eventually be found in other thymic tissue.

Normal (Lac x T6T6) F_1 mice were grafted under the kidney capsule with one lobe of a newborn CBA/H.T6T6 thymus, and one lobe of a newborn CBA/Lac thymus. The Lac thymus was placed at the anterior end of the kidney, and the T6T6 thymus was placed at the posterior end of the same kidney. Sixty five days after grafting the animals were killed and their thymus grafts were removed. Care was taken to keep the Lac and the T6T6 grafts separate. The thymus grafts were then teased, and the cells washed twice in medium 199, counted, and placed in culture with (Lac x T6T6) F_1 peripheral blood cells for 3 days with PHA. After harvesting, the metaphase plates were scored as being of Lac, T6T6 or (Lac x T6T6) F_1 origin. The F_1 mitotic figures were discarded from the results presented in Table 16. It can be seen in Lac thymus grafts that no cells of T6T6 origin could be found responding to PHA. The converse was true when T6T6 thymus grafts were examined. The conclusion to be drawn from

this experiment is that the persistent PHA-responsive cell population does not leave the thymus, circulate, and then return to the thymus. If this was the case one might have expected to find some cells of the "wrong" karyotype in the other thymus graft. Since the thymus grafts are syngeneic there is no reason to presume that a persistent PHA-responsive cell produced in one thymus graft would preferentially home to the thymus graft that produced it.

From the evidence presented in this and previous sections it would appear that the persistent PHA-responsive cell population is produced in the thymus, and stays in that environment for the rest of its life span. There is also no evidence to equate it with the population of cells that has been shown to home to spleen and/or lymph nodes on transfer (Raff, 1971; Lance et al., 1971).

TABLE 12

Flow Sheet of First Thymocyte Recirculation Experiment

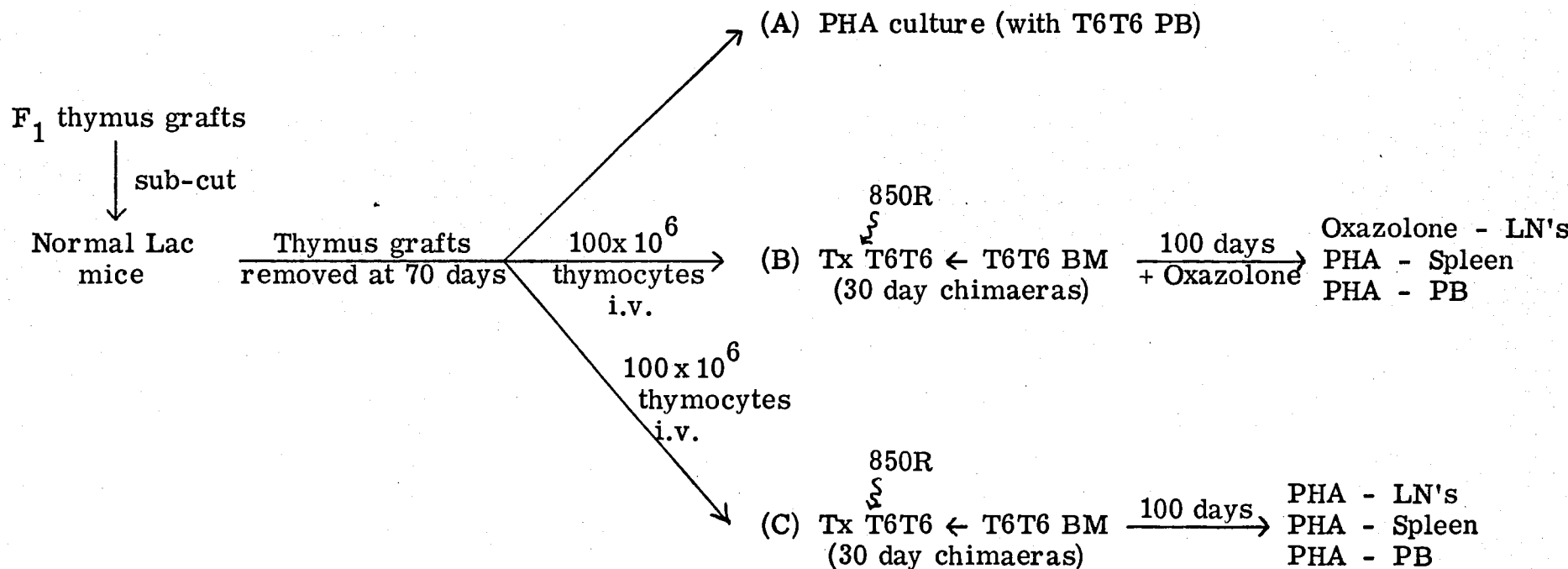


TABLE 13

The distribution of thymus graft cells injected into thymectomized radiation chimaeras (1st Experiment).

	Origin of thymus graft mitoses		Number of mitoses of thymus graft origin scored
	Thymus graft % F ₁ mitoses	Thymus graft recipient % Lac mitoses	
(A) Day 70 PHA culture	70.7	29.3	99
(B)* Day 170 Oxazolone - LN's	0.2	99.8	167
PHA - Spleen	0.6	99.4	121
PHA - PB	39.0	61.0	164
(C)* Day 170 PHA - LN's	6.1	93.9	90
PHA - Spleen	7.0	93.0	83
PHA - PB	31.7	68.3	128

* 3 mice per group

TABLE 14

Flow Sheet of Second Thymocyte Recirculation Experiment

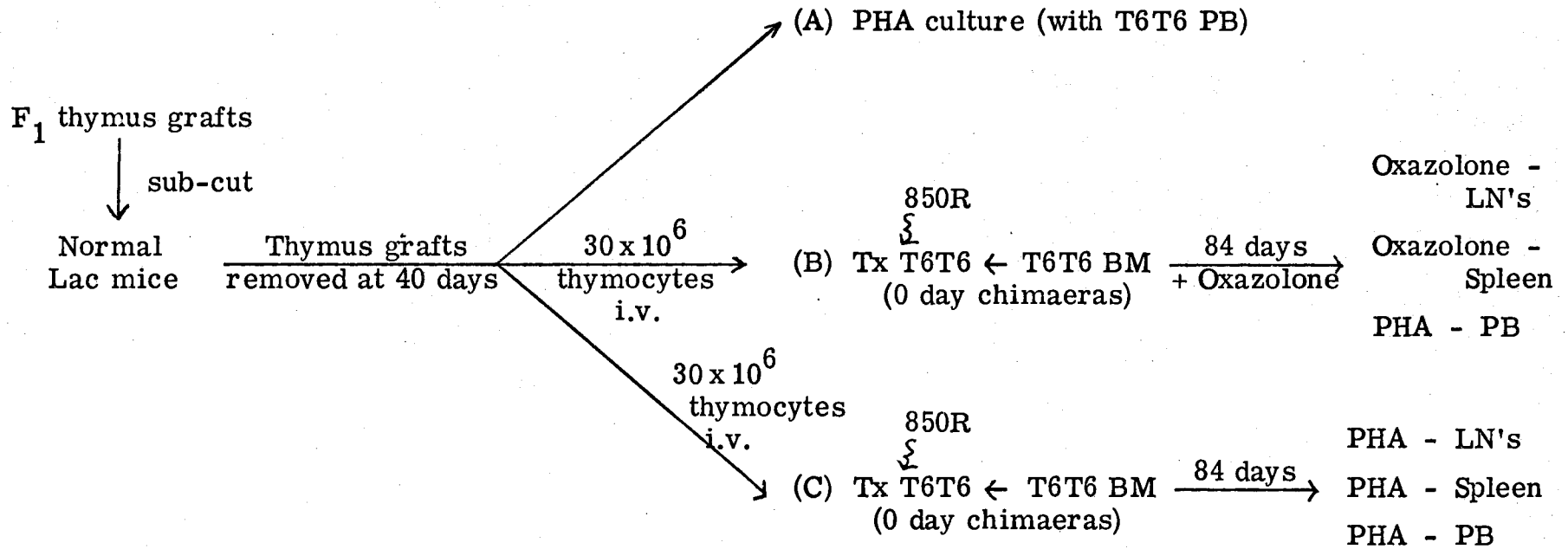


TABLE 15

The distribution of thymus graft cells injected into thymectomized radiation chimaeras (2nd Experiment).

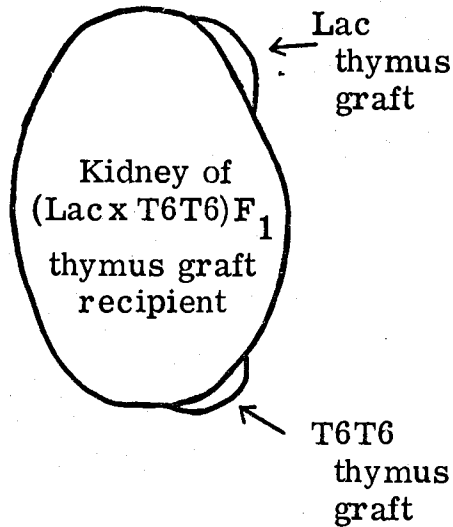
	Origin of thymus graft mitoses		Number of mitoses of thymus graft origin scored
	Thymus graft % F ₁ mitoses	Thymus graft recipient % Lac mitoses	
(A) Day 40 PHA culture	79.0	21.0	100
(B)* Day 124 Oxazolone - LN's	0.3	99.7	150
Oxazolone - Spleen	0	100	58
PHA - PB	43.5	56.5	50
(C)* Day 124 PHA - LN's	2.0	98.0	88
PHA - Spleen	4.9	95.1	158
PHA - PB	46.5	53.5	148

* 4 mice per group

TABLE 16

The percentage of PHA-responsive cells of T6T6 or Lac type in T6T6 or Lac thymus grafts, 65 days after grafting into (Lac x T6T6) F_1 mice.

Experimental Scheme *



% Lac **

% T6T6

100

0

0

100

* 4 animals examined and an average of 39 cells of thymus graft origin were scored in each thymus graft.

** The thymus graft cells were cultured with (Lac x T6T6) F_1 peripheral blood lymphocytes.

IMMUNOLOGICAL PROPERTIES OF PERSISTENT PHA-
RESPONSIVE CELLS IN MOUSE THYMUS GRAFTS

The thymus contains cells which, when teased out of the organ, have various properties. A proportion of them can respond to PHA (Colley et al., 1970; Blomgren and Svedmyr, 1971; Elliott et al., 1971); whole thymocyte populations transferred into irradiated adult or neonatally thymectomized mice confer some restoration of immunological function (Transplantation Reviews 1, 1969); thymocytes can be activated by a variety of antigenic stimuli; for example by passage into irradiated animals along with antigen they can specifically cooperate with B-cells in augmenting the production of humoral antibody (Andersson and Blomgren, 1970); they can be used to initiate graft-versus-host reactions (Blomgren and Andersson, 1969), mixed lymphocyte reactions (Knight and Thorbecke, 1971), and to destroy tumour cells (Cerottini et al., 1970).

With these immunological properties of thymus cells in mind, it was of interest to see if the persistent PHA-responsive cell population was synonymous with the population of cells in the thymus that could initiate immune responses. It seemed from the results presented in the previous section, that these cells did not home very well to peripheral lymphoid tissues when injected into

deprived recipients, and very few "persistent" cells could be shown responding to the skin sensitizing agent oxazolone in draining lymphoid organs. These experiments were undertaken to see if the persistent PHA-responsive cell population could proliferate in response to allogeneic stimulation, both in the GvH and the MLR reactions.

(A) GvH potential of persistent PHA-responsive cells

In the experiment outlined in Table 17, 20 newborn CBA/H.T6T6 thymuses were grafted subcutaneously into each normal (Lac x T6T6)F₁ mouse. Ninety seven days later the mice were injected intraperitoneally with 10 mg hydrocortisone sodium succinate. Three days later the mice were killed and their thymus grafts were removed, teased, and the cells obtained were washed several times in medium 199. The cell yield was less than 10% of that expected if hydrocortisone had not been injected. Some of the cells were placed in culture with PHA for 3 days, while the rest were injected into lethally irradiated (850R) (BALB/c x CBA/Lac) F₁ mice. Each mouse received 20×10^6 hydrocortisone-treated thymus graft cells intravenously. Four days later (a time arrived at after much trial and error) the mice were injected with colcemid, and 75 minutes later they were killed and their spleens removed for cytological analysis. A control group that had been

left uninjected was included, and both groups of animals had their body weights and spleen weights recorded. The spleen index (Simonsen et al., 1958) was 1.65 in the mice injected with thymus graft cells, indicative of a GvH reaction. When the mitotic population of cells taking part in the GvH reaction was examined (Table 17), it could be seen that there were no cells of recipient type (BALB/c x Lac) responding, and that of the thymus graft cells responding, 100% ($216/216$) were of thymus graft recipient type (T6T6 x Lac). No cells of the thymus graft donor type (T6T6 - "persistent") were seen responding, even though 77.4% of the cells responding to PHA were of donor karyotype.

One interpretation of these results is that the persistent PHA-responsive cell population is not responsive to allogeneic stimulus. It would be unfair to come to such a conclusion however, since it was shown in the previous section of this thesis that the "persistent" population did not recirculate to spleen and lymph nodes, and as such would be unlikely to home to the spleen in a GvH assay. Thus, no firm conclusion could be made from this experiment, on the immunological capabilities of the persistent PHA-responsive cell population, but it could reasonably be concluded that they played little or no part in the GvH response in the spleen of recipient animals.

(B) MLR reactivity of the "persistent" population

Since it has been shown that the "persistent" population did not respond mitotically to oxazolone or to allogeneic stimulation (GvH) perhaps because these cells do not localize in sites where mitotic activity was assessed, an attempt was made to see if these cells could respond to allogeneic stimulation in vitro (MLR).

Multiple newborn CBA/H.T6T6 thymuses were grafted subcutaneously into normal (Lac x T6T6) F_1 mice. At either 60 days post grafting (Table 18, Expt. 1) or 97 days post grafting (Table 18, Expt. 2), the mice were injected intraperitoneally with 10 mg hydrocortisone sodium succinate. Three days later the mice were killed and their thymus grafts were removed, teased, and the cells obtained were washed several times in medium 199. Some of the cells were placed in culture with PHA for 3 days, while others were placed in culture with various types of allogeneic spleen cells. The allogeneic cells used were BALB/c, (BALB/c x CBA/Lac) F_1 and BALB/c mitomycin C treated spleen cells. From preliminary experiments it was seen that maximal mitotic responsiveness occurred when 4×10^6 thymocytes were mixed with 4×10^6 allogeneic spleen cells in 4 ml of tissue culture medium, and harvested at 5 days after initiation of the culture.

These conditions were thus used in the experiments reported in Table 18.

It can be seen in Table 18 that a substantial percentage of cells responding were of thymus graft origin in both experiments. Thus it would appear that certain thymus graft cells can respond mitotically to allogeneic cells in vitro. One surprising feature of the results was that there was a good mitotic response of (BALB/c x CBA/Lac) F₁ spleen cells to CBA thymus graft cells in vitro. One would expect BALB/c spleen cells to recognize CBA thymus graft cells as foreign, but would not expect an F₁ cell population to respond to the parental population. There is as yet no clear explanation of this phenomenon, although it has been demonstrated previously (Doenhoff, personal communication) in this laboratory. When BALB/c spleen cells were pre-incubated with Mitomycin C, they failed to respond to the CBA transplantation antigens on the thymus graft cells. This was the only instance in which a one-way mixed lymphocyte reaction could be demonstrated, and in all the other results presented, a two-way mixed lymphocyte reaction was in operation.

When the origin of the thymus graft cells taking part in the MLR was determined, it could be seen that the majority, in all cases, were of thymus graft recipient type (~65-79%), whereas

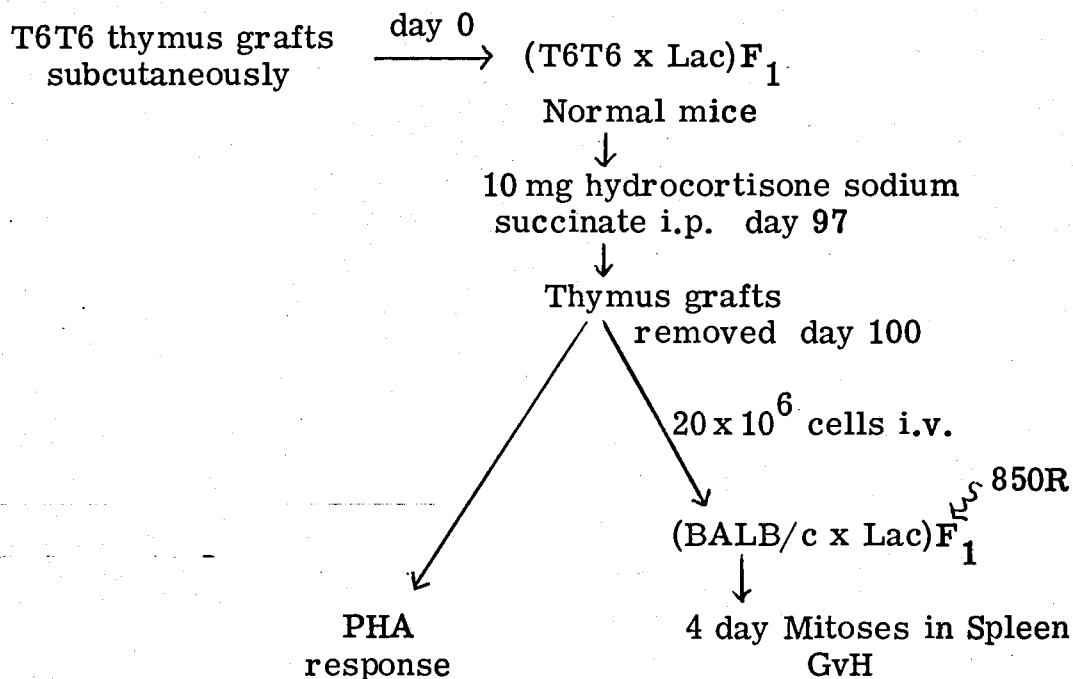
the converse was true of the PHA response ($\sim 23-29\%$). If the results of the mixed lymphocyte reactions and PHA responses are averaged, it can be calculated that in relation to the other cell population, either the "non-persistent" thymus graft population is 9.2 times more reactive to transplantation antigens than to PHA, or the "persistent" thymus graft population is 9.2 times less reactive to transplantation antigens than to PHA.

Although no firm conclusions can be drawn from these experiments, it is tempting to suggest that the persistent PHA-responsive cell population has relatively little reactivity to transplantation antigens in vitro. With more and more reports in the literature suggesting that the products of activated cell populations can stimulate proliferation in other non-activated cell populations (Piguet and Vassalli, 1972; Andersson et al., 1972; Cerny, 1974; Hunter and Kettman, 1974), it is feasible that products produced by cells responding to histocompatibility antigens could in fact stimulate proliferation in a non-responding population of cells. This might explain why (BALB/c x CBA/Lac) F_1 cells can be found in mitosis when mixed with a parental cell population, and why some cells at least of the persistent PHA-responsive cell population appear to participate in mixed lymphocyte reactions.

Although it has not conclusively been demonstrated here that the persistent PHA-responsive cell population has no immunological reactivity, it has been shown that it is slight in comparison to that of the "non-persistent" population. There is confirmatory evidence in the literature which would tend to support this notion. Both Colley et al. (1970) and Knight et al. (1973) showed that the population of cells in the rat thymus which took part in mixed lymphocyte reactions were not identical to those cells responding to PHA, when the thymocytes were separated on BSA gradients. These results again suggest that the majority of the PHA-responsive cell population is poorly responsive to allogeneic stimulation, and may be equated with the persistent PHA-responsive cell population found in mouse thymus grafts.

TABLE 17

Graft versus Host potential of the persistent PHA-responsive cell population.



Percent Mitoses

	T6T6 (thymus graft donor type)	(T6T6 x Lac) F_1 (thymus graft recipient type)
PHA	77.4	22.6
GvH*	0	100 ($^{216}/216$ cells counted)

* No (BALB/c x Lac) F_1 cells seen responding in a group of 4 animals tested.

TABLE 18

Reactivity of hydrocortisone-treated thymus graft cells*
 (T6T6 thymus graft → normal (T6T6 x Lac)F₁) in mixed
 lymphocyte reactions.

Stimulant	% Mitoses at 5 days**		% Mitotic response of thymocyte subpopulation in MLR & PHA response	
	Stimulator cells	Thymus graft cells	Thymus graft donor T6T6	Thymus graft recipient (T6T6 x Lac)F ₁
Expt. 1				
BALB/c Spleen cells*	50.0	50.0	24.0	76.0
BALB/c x Lac Spleen cells	51.0	49.0	18.4	71.6
PHA (3 day response)	—	—	71.0	29.0
Expt. 2				
BALB/c Spleen cells	39.5	60.5	34.7	65.3
BALB/c (Mitomycin C) Spleen cells	0	100	32.7	67.3
BALB/c x Lac Spleen cells	24.0	76.0	21.1	78.9
PHA (3 day response)	—	—	77.4	22.6

* Pooled cells

** Average of 89 mitoses scored per group

CONCLUDING DISCUSSION

In preceding sections of this thesis many characteristics of the PHA-responsive cell population in mouse thymus grafts have been described. Initially it was shown that this cell population(s) was derived from the thymus graft itself, presumably from stem cells present in it at the time of grafting, and not from a blood borne population of T-cells (Elliott et al., 1971). When, however, thymus grafts were examined at late times after grafting for the presence of PHA-responsive cells, it was shown that a majority of the cells responding were of native thymus graft type long after any evidence of their mitotic expression in the thymus could be demonstrated (Elliott, 1973). These cells were termed the "persistent PHA-responsive population".

Although over 70% of the cells in division, in response to PHA, were of the persistent type, no conclusion was drawn as to the actual numbers or percentage of these cells in the mouse thymus medulla, since different percentages were obtained when thymus graft cells were stimulated with other mitogens. It is probably reasonable to assume, however, that the persistent population does make up quite a large percentage of the lymphoid cells in the thymus medulla. It should be noted in these experiments that some cells were found that derived from the

host, and some at least of these cells could indeed be the mature cell population that is about to leave the thymus (Börum, 1968).

Further experiments showed that the persistent population was relatively insensitive to the effects of large doses of hydrocortisone, evidence that placed it in the medullary lymphocyte population (Warner, 1964; Dougherty et al., 1964; Ishidate and Metcalf, 1963).

One hypothesis considered for the function of the persistent population was that it was readily mobilizable, and in the event of any catastrophe to the peripheral T-cell population, could be readily released into the circulation to fill the space available. This hypothesis was tested by treating animals bearing thymus grafts with ALS. ALS causes a depletion of the peripheral T-cell pool, with little if any direct effect on the thymus (reviewed by Lance et al., 1973). Under these circumstances, using a potent ALS, no change in the percentage of persistent PHA-responsive cells was noted, thus invalidating this particular hypothesis.

Experiments were designed to see if the persistent population could be demonstrated in any situation other than when syngeneic thymuses were grafted. In the instance of AK thymus grafts in deprived CBA mice, a large percentage of the PHA-

responsive cells at late times after grafting were of the foreign thymus graft type. Furthermore, repopulation studies of irradiated thymuses in situ showed that persistent PHA-responsive cells could be found. This evidence is suggestive of the fact that persistent cells would be found in the normal adult thymus if only other methods for their detection could be discovered.

Using an anti- θ_{AKR} serum and complement, it was shown that the persistent population in allogeneic thymus grafts were θ -sensitive. Owen and Raff (1970) hinted at the presence of the persistent cell population when they showed that over 3% of the cells in allogeneic thymus grafts, at 42 days post grafting, were of thymus graft type. These cells also possessed the θ -antigen. Thus, so far, the persistent population has been shown to possess two characteristics of the peripheral T-cell population, namely PHA-responsiveness and the possession of the θ -antigen, yet despite these similarities these two populations should not be equated.

When thymus graft cells were sedimented on a 0.2-2% BSA gradient, further evidence was provided for the heterogeneity of the PHA-responsive populations. Although separation was not complete, there was some distinction between those PHA-responsive cells bearing the karyotype of the thymus graft donor,

and those PHA-responsive cells bearing the karyotype of the thymus graft recipient. The persistent PHA-responsive population tended to consist of larger cells than the non-persistent PHA-responsive population, and due to the fact that some non-persistent cells were found even at the bottom end of the gradient, would suggest that some cells at least showing the non-persistent karyotype might persist in the thymus, while others might comprise the population that is about to leave the thymus.

There is now much evidence to suggest that the stem cells responsible for the production of the peripheral T-cell population are derived from yolk sac and foetal liver in the embryo, and from the bone marrow in the adult animal (reviewed by Owen, 1972). Due to striking similarities between the peripheral T-cell population and the persistent population in the thymus (viz. PHA responsiveness and θ sensitivity), many attempts were made to determine if the latter also was derived initially from a stem cell that entered the thymus from sources such as bone marrow, and not from thymic epithelial elements, as Auerbach (1961) suggested for certain thymic lymphocytes. The experiments performed, although not conclusive, can be interpreted in all instances as indicating that these persistent cells did in fact originate from stem cells that initially derived from sources external to the

thymus, but whether the stem cell responsible for peripheral T-cells and persistent cells is the same cell, has yet to be demonstrated.

Although the persistent PHA-responsive cell population was shown not to leave the thymus, experiments were performed to determine its behavioural characteristics, when deliberately injected, as a cell suspension, into deprived mice. It has long been known that T-cells can recirculate from blood-lymph-blood (Gowans, 1959; Gowans and Knight, 1965), and since the persistent population shows certain similarities to the peripheral T-cell population it was of interest to determine if it had the same recirculatory characteristics. Although no early localization studies were performed, it was shown that by 84 and 100 days after injection, very few cells of persistent type could be found in the spleen and lymph nodes, but a much higher percentage could be found in the peripheral blood. Thus it seemed that the persistent population could circulate in the peripheral blood but not recirculate (in the Gowans' sense) through spleen and lymph nodes, and so could not be equated with the peripheral T-cell population.

When certain immunological properties of the persistent population were examined, no response to either the skin sensitizing

agent oxazolone, or to foreign histocompatibility antigens (GvH) could be detected. This was not surprising since it had previously been shown that these cells could be found in only small numbers in recipient spleens. Finally it was shown that the persistent population was very poor in its mitotic responsiveness to foreign cells in the mixed lymphocyte reaction. It appeared to respond much less well to foreign cells than to PHA, when compared to the population of cells with the non-persistent karyotype.

From these studies and those of other workers (Colley et al., 1970; Knight et al., 1973), it appears that in the thymic medulla there are at least two types of lymphocyte. Both these cell populations can respond to PHA, are θ -positive, and appear to be larger than the majority of cortical lymphocytes. Where they differ however, is in their immunological and recirculatory characteristics. The persistent PHA-responsive population cannot localize in spleen or lymph nodes, and has limited immunological reactivity, whereas the majority of cells with the non-persistent karyotype would appear to be able to recirculate and respond in the graft versus host and mixed lymphocyte reactions.

If the persistent population shows little or no immunological

responsiveness to antigens, and does not leave the thymus, what function has it? It was shown earlier that it was not a readily mobilizable T-cell population which could be liberated when disaster struck the peripheral T-cell pool. One other popular function attributed to the thymus is the production of thymus humoral factor (thymosin), but as yet there is no evidence to link the persistent population with the production of thymus hormone. Indeed, circumstantial evidence would tend to rule this out. Firstly, it would appear that the cells which produce this factor are found in the thymus epithelium (Mandi and Glant, 1973; Vettors and Macadam, 1973), and secondly, restoration of "thymus hormone like" activity in the serum of thymus-grafted mice occurs long before the persistent population can be demonstrated (Bach and Dardenne, 1973).

As no positive immunological function could be demonstrated for the persistent population, perhaps it could exert a negative immunological function. In the middle nineteen-sixties, Waksman and his co-workers showed that immunologically deprived rats, when injected with normal syngeneic bone marrow cells and a thymus graft from animals previously tolerized with BGG, failed to respond when injected later with BGG. They concluded that specific immunological tolerance could be induced in the thymus,

and that tolerance could be transferred to other animals by thymus grafts (Isakovic, Smith and Waksman, 1965; Smith, Isakovic and Waksman, 1966). Later, after the discovery that T-cells could cooperate with B-cells in the formation of antibody (Claman et al., 1966; Davies et al., 1967; Mitchell and Miller, 1968), Weigle and his co-workers showed in an adoptive transfer system, that thymuses removed from animals tolerant to deaggregated HGG could not cooperate with B-cells in the production of antibodies to HGG. This state of immunological anergy in the thymocyte population was long lasting whereas tolerance in the B-cell precursor population (bone marrow) was transient (reviewed by Weigle, Chiller and Habicht, 1972). More recently a rapidly growing number of phenomena have suggested that there are cells in the thymus which can suppress immune responses when transferred to recipient animals (Droege, 1971; Gershon et al., 1972; Ha and Waksman, 1973). These cells are termed "suppressor T-cells" (reviewed by Gershon, 1973). Furthermore, Ha, Waksman and Treffers (1974) have suggested that these "suppressor" cells in the thymus reside in "a subpopulation of low density, making up less than 10% of the total thymocytes, and are partially resistant to hydrocortisone".

It is postulated that some of the phenomena described

above could be carried out by the persistent population, cells of which have low positive immunological activity, but may exert a controlling influence on the immunological phenomena that are allowed to occur within the thymus. Of course this hypothesis can only be tested when almost complete separation is obtained of the various sub-populations found in the thymus.

A question may be asked why the persistent population had not been demonstrated previously by other workers? Apart from the hint of persistent cells in the results of Owen and Raff (1972) and Cheers et al. (1972), no such demonstration has been made. Experiments on electrophoretically separated thymocytes have suggested that the thymus does consist of more than two cell subpopulations (Nordling et al., 1972; Zeiller and Dolan, 1972; Wiig, 1973) but as yet none of these cell populations so separated have been associated with persistence in the thymus. Even in the elegant radioactive thymidine labelling experiments of Børum (1968), no labelled lymphoid cells were seen to persist for long periods in the thymus. The most likely explanation of these results and those of other workers (Weissman, 1967; Sainte-Marie and Leblond, 1964a; Parrott and de Sousa, 1967), is that the thymuses were labelled at a time when the persistent population had already been formed, and hence could not be

further labelled. Perhaps the study that came closest to the discovery of the persistent population was that of Cheers et al. (1972). These workers showed that only a small proportion of the cells responding to oxazolone were of native thymus graft type, when thymus graft cells were transferred to deprived recipients at late times after grafting (viz. 50 and 100 days). As will be noted from previous sections of this thesis, the persistent population does not localise in lymph nodes but remains in the peripheral blood. If Cheers and her coworkers had only examined the peripheral blood of their chimaeras, they might have shown a considerable proportion of native thymus graft cells responding to PHA.

In 1962 Burnet expressed an opinion on work concerned with the function of the thymus. He said "we are taking part in the elucidation of the function of the last organ of the body to remain a mystery". It is hoped that the work presented in the preceding sections of this thesis does not add too much to the mystery surrounding the function of the thymus.

REFERENCES

- Adler, W.H. personal communication.
- Aird, J. (1971) M.Phil. thesis, University of London.
- Andersson, B. and Blomgren, H. (1970) Cell.Immunol. 1, 362.
- Andersson, J., Möller, G. and Sjöberg, O. (1972) Eur.J. Immunol. 2, 99.
- Andreasen, E. and Christensen, S. (1949) Anat.Rec. 103, 401.
- Auerbach, R. (1960) Develop.Biol. 2, 271.
- Auerbach, R. (1961) Develop.Biol. 3, 336.
- Auerbach, R. (1963) Nat. Cancer Inst. Monograph 11, 23.
- Auerbach, R. (1964) In "The Thymus in Immunobiology" ed. R.A. Good and A.E. Gabrielson. Harper and Row, New York.
- Bach, J-F. and Dardenne, M. (1973) Immunology 25, 353.
- Bach, J-F. (1971) In "In vitro Correlates of Cell Mediated Immunity", S. Karger, Basel.
- Blomgren, H. and Andersson, B. (1969) Exp. Cell Res. 57, 185.
- Blomgren, H. and Svedmyr, E. (1971) Cell.Immunol. 2, 285.
- Borjeson, J., Reisfield, R., Chessin, L.N., Welsh, P. and Douglas, S.D. (1966) J. Exp. Med. 124, 859.
- Borum, K. (1965) Nature 208, 253.
- Borum, K. (1968) Scand. J. Haematol. 5, 339.
- Borum, K. (1973) Cell Tissue Kinet. 6, 545.

- Boyse, E.A., Miyazawa, M., Aoki, T. and Old, L.J. (1968)
Proc. Roy. Soc. B 170, 175.
- Boyse, E.A., Old, L.J. and Luell, S. (1963) J. Nat. Cancer
Inst. 31, 987.
- Boyse, E.A., Old, L.J. and Stockert, E. (1965) In "IVth Int.
Symp. Immunopathology" Ed. P. Grabar and P.A.
Miescher. Schwabe, Basel.
- Boyse, E.A. and Old, L.J. (1969) Ann. Rev. Genet. 3, 269.
- Burnet, F.M. (1962) Brit. Med. J. 2, 807.
- Cantor, H. and Asofsky, R. (1973) 243, 39.
- Cerny, J. (1974) Nature 249, 63.
- Cerottini, J-C., Nordin, A.A. and Brunner, K.T. (1970)
Nature 227, 72.
- Cheers, C., Leuchars, E., Wallis, V. and Davies, A.J.S.
(1972) Transplantation 13, 72.
- Claman, H.N. (1966) Proc. Soc. Exp. Biol. Med. 121, 236.
- Claman, H.N., Chaperon, E.A. and Triplett, R.F. (1966)
Proc. Soc. Exp. Biol. Med. 122, 1167.
- Cohen, A. and Schlesinger, M. (1970) Transplantation 10, 130.
- Colley, D.G., Shih Wu, A.Y. and Waksman, B.H. (1970)
J. Exp. Med. 132, 1107.
- Davies, A.J.S., Leuchars, E., Wallis, V. and Koller, P.C.
(1966) Transplantation 4, 438.
- Davies, A.J.S., Leuchars, E., Wallis, V., Marchant, R. and
Elliott, E.V. (1967) Transplantation 5, 222.

- Davies, A.J.S. (1969) *Transplant.Rev.* 1, 43.
- Davies, A.J.S., Leuchars, E., Wallis, V. and Doenhoff, M.J.
(1971) *Proc.Roy.Soc.Lond.B* 176, 369.
- Doenhoff, M.J. personal communication.
- Doenhoff, M.J. (1970) Ph.D. thesis, University of London.
- Doenhoff, M.J., Davies, A.J.S., Leuchars, E. and Wallis, V.
(1970a) *Proc.Roy.Soc.Lond.B* 176, 69.
- Doenhoff, M.J., Davies, A.J.S., Leuchars, E. and Wallis, V.
(1970b) *Nature* 227, 1352.
- Doenhoff, M.J. (1971) *Clin.Exp.Immunol.* 8, 603.
- Dougherty, T.F. (1952) *Phys.Rev.* 32, 379.
- Dougherty, T.F., Berliner, M.L., Schneebeli, G.L. and
Berliner, D.L. (1964) *Ann.N.Y.Acad.Sci.* 117, 825.
- Droege, W. (1971) *Nature* 234, 549.
- Dukor, P., Miller, J.F.A.P., House, W. and Allman, V. (1965)
Transplantation 3, 639.
- Elliott, E.V., Wallis, V. and Davies, A.J.S. (1971) *Nature New
Biol.* 234, 77.
- Elliott, E.V. (1973) *Nature New Biol.* 242, 150.
- Ernström, U., Gyllensten, L. and Larsson, R. (1965) *Nature*
207, 540.
- Ernström, U. and Larsson, R. (1967) *Acta Path.Microbiol.
Scand.* 70, 371.
- Ernström, U. and Larsson, R. (1969) *Nature* 222, 279.

- Everett, N.B. and Tyler, R.W. (1967) *Int.Rev.Cytology* 22, 205.
- Festenstein, H. (1968) *Lancet* 1, 182.
- Ford, C.E. (1966) In "Tissue Grafting and Radiation" Academic Press, New York.
- Gershon, R.K., Cohen, P., Henan, R. and Liebhaber, S.A. (1972) *J.Immunol.* 108, 586.
- Gershon, R.K. (1973) *Contemp.Top.Immunobiol.* 3, 1.
- Gordon, H.A. (1959) *Ann.N.Y.Acad.Sci.* 78, 208.
- Gowans, J.L. (1959) *J.Physiol.* 146, 54
- Gowans, J.L. and Knight, E.J. (1965) *Proc.Roy.Soc.B* 159, 257.
- Greaves, M.F., Owen, J.J.T. and Raff, M.C. (1973) T and B Lymphocytes: Origins, properties and roles in immune responses. Excerpta Medica, Amsterdam.
- Greaves, M. and Janossy, G. (1972) *Transplant.Rev.* 11, 87.
- Ha, T-Y. and Waksman, B.H. (1973) *J.Immunol.* 110, 1290.
- Ha, T-Y., Waksman, B.H. and Treffers, H.P. (1974) *J.Exp.Med.* 139, 13.
- Harris, J.E., Ford, C.E., Barnes, D.W.H. and Evans, E.P. (1964) *Nature* 201, 886.
- Hays, E.F. (1969) *J.Exp.Med.* 129, 1235.
- Hunter, P. and Kettman, J.R. (1974) *Proc.Nat.Acad.Sci.USA* 71, 512.
- Isakovic, K., Smith, S.B. and Waksman, B.H. (1965) *J.Exp.Med.* 122, 1103.

- Ishidate, M. and Metcalf, D. (1963) *J. exp. Biol.* 41, 637.
- Jacobsson, H. and Blomgren, H. (1972) *Cell. Immunol.* 5, 107.
- Kerbel, R.S., Elliott, E.V. and Wallis, V. (1974) *Cell. Immunol.* 11, 146.
- Knight, S.C. and Thorbecke, G.J. (1971) *Cell. Immunol.* 2, 91.
- Knight, S.C., Newey, B. and Ling, N.R. (1973) *Cell. Immunol.* 9, 273.
- Koller, P.C., Davies, A.J.S., Leuchars, E. and Wallis, V. (1966) In "The Lymphocyte in Immunology and Haemopoiesis" p.342, Edward Arnold.
- Konda, S., Nakao, Y. and Smith, R.T. (1972) *J. Exp. Med.* 136, 1461.
- Lance, E.M. (1969) *J. Exp. Med.* 130, 49.
- Lance, E.M., Cooper, S. and Boyse, E.A. (1971) *Cell. Immunol.* 1, 536.
- Lance, E.M., Medawar, P.B. and Taub, R.N. (1973) *Adv. Immunol.* 17, 1.
- Leckband, E. (1970) *Fed. Proc.* 29, 621.
- Leckband, E. and Boyse, E.A. (1971) *Science* 172, 1258.
- Leuchars, E. (1966) Ph.D. thesis, University of London.
- Leuchars, E., Morgan, A., Davies, A.J.S. and Wallis, V.J. (1967) *Nature* 214, 801.
- Leuchars, E., Aird, J., Davies, A.J.S. and Wallis, V. (1970) In "Protides of the Biological Fluids", p.73, Pergamon Press, Oxford and New York.

- Levey, R.H. and Medawar, P.B. (1966) Proc. Nat. Acad. Sci. USA 56, 1136.
- Lunden, P.M. (1958) Acta Endocrin. 28, Suppl. 40.
- Luria, E.A. and Domashneva, I.V. (1974) Proc. Nat. Acad. Sci. 71, 235.
- McIntyre, O.R. and Segel, V.D. (1966) Lancet 1, 1265.
- Mandi, B. and Glant, T. (1973) Nature New Biol. 246, 25.
- Metcalf, D. (1964) In "The Thymus in Immunobiology", ed. R.A. Good and A.E. Gabrielson, Harper and Row, New York.
- Metcalf, D. (1966) In "The Thymus: Experimental and Clinical Studies," CIBA Foundation Symposium, ed. G.E.W. Wolstenholme and R. Porter.
- Metcalf, D. and Moore, M.A.S. (1971) "Haematopoietic Cells: their origin, migration and differentiation" (Frontiers of Biology, 24), North-Holland, Amsterdam.
- Metcalf, W.K. (1966) In "Lymphocytes in Immunology and Haemopoiesis" (J.M. Yoffey, ed.) p. 62, Arnold, London.
- Micklem, H.S., Ford, C.E., Evans, E.P. and Gray, J. (1966) Proc. Roy. Soc. B 165, 78.
- Micklem, H.S. and Loutit, J.F. (1966) In "Tissue Grafting and Radiation" Academic Press, New York.
- Micklem, H.S., Ogden, D.A. and Pritchard, H. (1972) Clin. Exp. Immunol., 12, 103.
- Miller, J.F.A.P. (1960) Brit. J. Cancer 14, 93.

- Miller, J.F.A.P. (1962) Proc. Roy. Soc. B 156, 415.
- Miller, J.F.A.P. and Davies, A.J.S. (1964) Ann. Rev. Med. 15, 23.
- Miller, J.F.A.P., de Burgh, P.M., Dukor, P., Grant, G.,
Allman, V. and House, W. (1966) Clin. Exp. Immunol.
1, 61.
- Miller, J.F.A.P. (1967) Lancet ii, 1299.
- Miller, J.F.A.P. and Osoba, D. (1967) Phys. Rev. 47, 437.
- Miller, R.G. and Phillips, R.A. (1969) J. Cell. Physiol. 73, 191.
- Mitchell, G.F. and Miller, J.F.A.P. (1968) J. Exp. Med. 128, 821.
- Modabber, F.I., Morikawa, S. and Coons, A.H. (1967) Science
170, 1102.
- Moore, M.A.S. and Owen, J.J.T. (1967a) Lancet ii, 658.
- Moore, M.A.S. and Owen, J.J.T. (1967b) J. Exp. Med. 126, 715.
- Nordling, S., Andersson, L.C. and Häyry, P. (1972) Eur. J.
Immunol. 2, 405.
- Owen, J.J.T. and Ritter, M. (1969) J. Exp. Med. 129, 431.
- Owen, J.J.T. and Raff, M.C. (1970) J. Exp. Med. 132, 1216.
- Owen, J.J.T., Hunter, P. and Raff, M.C. (1971) Transplantation
12, 231.
- Owen, J.J.T. (1972) In "Ontogeny of Acquired Immunity" Ciba
Foundation Symposium, Elsevier, Excerpta Medica,
North-Holland, Amsterdam - London - New York.
- Parrott, D.M.V. and De Sousa, M.A.B. (1967) Immunology
13, 193.

- Piguet, P-F. and Vassalli, P. (1972) *J. Exp. Med.* 136, 962.
- Poste, M.E. and Olson, I.A. (1973) *Immunology* 24, 691.
- Raff, M.C. (1971a) *Nature* 229, 182.
- Raff, M.C. (1971b) *Transplant. Rev.* 6, 52.
- Raff, M.C. and Owen, J.J.T. (1971) In "Morphological and Functional Aspects of Immunity" ed. K. Lindahl-Kiessling, G. Alm and M.G. Hanna. Plenum Press, New York.
- Raviola, E. and Karnovsky, M.J. (1972) *J. Exp. Med.* 136, 466.
- Reif, A.E. and Allen, J.M.V. (1963) *Nature* 200, 1332.
- Reif, A.E. and Allen, J.M.V. (1964) *J. Exp. Med.* 120, 413.
- Ritter, M. (1972) D.Phil. thesis, Oxford University.
- Roitt, I.M., Greaves, M.F., Torrigiani, G., Brostoff, J. and Pläyfair, J.H.L. (1969) *Lancet* ii, 367.
- Sainte-Marie, G. and Leblond, C.P. (1964a) In "The Thymus in Immunobiology" ed. R.A. Good and A.E. Gabrielson, Harper and Row, New York.
- Sainte-Marie, G. and Leblond, C.P. (1964b) *Blood* 23, 275.
- Sainte-Marie, G. and Leblond, C.P. (1965) *Blood* 26, 765.
- Sarton, G. (1927) In "Introduction to the History of Science" Baltimore Press.
- Schlesinger, M. (1972) *Progr. Allergy* 16, 214.
- Schrek, R. and Batra, K.V. (1966) *Lancet* 2, 444.
- Shortman, K., Brunner, K.T. and Cerottini, J-C. (1972a) *J. Exp. Med.* 135, 1375.

- Shortman, K., Cerottini, J-C. and Brunner, K. T. (1972b)
Eur. J. Immunol. 2, 313.
- Simonsen, M., Engelbreth-Holm, J., Jensen, E. and Poulsen, H.
(1958) Ann. N. Y. Acad. Sci. 73, 834.
- Smith, S. B., Isakovic, K. and Waksman, B. H. (1966) Proc. Soc.
Exp. Biol. Med. 121, 1005.
- Statny, P. and Ziff, M. (1966) Arthritis Rheumat. 9, 543.
- Transplantation Reviews (1969) 1, Munksgaard, Copenhagen.
- Tyan, M. L. (1968) J. Immunol. 100, 535.
- Venzke, W. G. (1952) Am. J. Vet. Res. 13, 395.
- Vetters, J. M. and Macadam, R. F. (1973) J. Clin. Path. 26, 194.
- Vitetta, E. S., Uhr, J. W. and Boyse, E. A. (1973) Proc. Nat.
Acad. Sci. 70, 834.
- Wallis, V. J., Leuchars, E., Chwalinski, S. and Davies, A. J. S.
(1975) Transplantation (in press).
- Warner, N. L. (1964) Aust. J. Exp. Biol. Med. Sci. 42, 401.
- Weber, W. T. (1966a) J. cell Physiol. 67, 285.
- Weber, W. T. (1966b) J. cell Physiol. 68, 117.
- Weber, W. T. (1970) Clin. exp. Immunol. 6, 919.
- Weber, W. T. (1967) Exp. Cell Res. 46, 464.
- Weigle, W. O., Chiller, J. M. and Habicht, G. S. (1972) Transplant.
Rev. 8, 3.
- Weissman, I. (1967) J. Exp. Med. 126, 291.

- Wiig, J.N. (1973) *Scand.J.Immunol.* 2, 23.
- Winkelstein, A. and Craddock, C.G. (1967) *Blood* 29, 594.
- Winn, H.J. (1960) *Nat.Cancer Inst.Monograph* 2, 113.
- Winn, H.J. (1962) *Ann.N.Y.Acad.Sci.* 101, 23.
- Wu, A.M., Till, J.E., Siminovitch, L. and McCulloch, E.A.
(1968) *J.Exp.Med.* 127, 455.
- Yoshida, T.O. and Andersson, B. (1972) *Scand.J.Immunol.*
1, 401.
- Yukotu, M., Grossberg, A.L. and Pressman, D. (1974)
J.Immunol. 112, 1774.
- Zeiller, K. and Dolan, L. (1972) *Eur.J.Immunol.* 2, 439.
- Zeiller, K., Pascher, G., Wagner, G., Liebich, H.G., Halzberg, E.
and Hannig, K. (1974) *Immunology* 26, 995.