Thymus

Experimental and Clinical Studies

A Ciba Foundation Symposium

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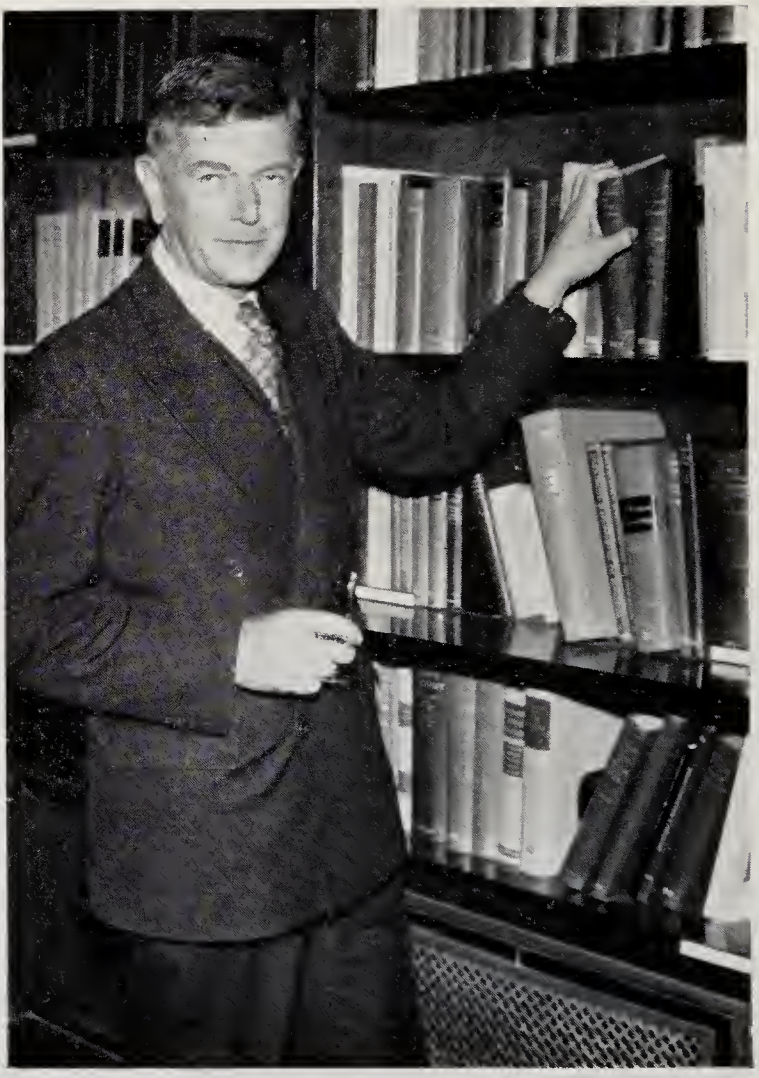
THE THYMUS
Experimental and Clinical Studies



Sir Macfarlane Burnet, O.M.

Guest of honour and Chairman

Ciba Foundation Symposium

THE THYMUS: EXPERIMENTAL AND CLINICAL STUDIES

In honour of SIR MACFARLANE BURNET, O.M.

Edited by G. E. W. WOLSTENHOLME, O.B.E., M.A., F.R.C.P., F.I.Biol.

and

RUTH PORTER, M.R.C.P.

With 96 illustrations

1966

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Preface

MOST of the Ciba Foundation's small international conferences are held at its house in Portland Place, London, but approximately every one to two years a symposium is organized in another part of the world. Such meetings were first held abroad in 1959 in Italy, France and the Argentine, and subsequently again in Italy, and in Spain, Egypt and Ethiopia. These occasional meetings overseas are partly to "show the flag" and to demonstrate the kind of meeting which we believe to be of value. They also permit us to vary the national, regional or language-speaking basic membership which, in London, is often but not invariably disproportionately prejudiced in favour of people working in Great Britain.

The present book represents an event of particular note among these overseas conferences. The symposium was arranged in honour of its Chairman, Sir Macfarlane Burnet, O.M., on his retirement as Director of the Walter and Eliza Hall Institute of Medical Research. For forty years Sir Macfarlane had served the Hall Institute—twenty-one years as its Director—during which time it has achieved a world reputation, first in virology and then, in a dramatic change introduced by Sir Macfarlane, in immunology.

The symposium also coincided with celebrations of the fiftieth anniversary of the founding of the Hall Institute, and the Ciba Foundation was honoured and flattered to be asked to join in a leading way in these important Australian occasions. The Government of Australia, through the Prime Minister's department, and the Australian Academy of Sciences (from the Becker Fund) gave substantial and generous contributions towards the cost of local facilities and members' travelling expenses. Sir Macfarlane himself made a most important grant from Royalties on his

publications so that with this, his inception of the meeting, and his inspiring chairmanship, he put us still further in his debt—further, because we are already deeply grateful to him for his support of the Ciba Foundation throughout its formative years.

Sir Macfarlane's successor, Professor G. J. V. Nossal, and his colleagues and members of the Hall Institute, spared no effort in securing the efficiency of the arrangements and the distinction and friendliness of the public and social events. We are also most indebted to Professor S. D. Rubbo for allowing us to hold the symposium in the new School of Microbiology—happily in the "Harold Woodruff" Lecture Theatre, since Professor Michael Woodruff was a member of the symposium and his father, Harold Woodruff, had been a close friend of Dr. Wolstenholme's parents.

We take great pleasure in acknowledging the help of Dr. Jacques Miller in the editing of this book. Dr. Miller's collaboration has been most appropriate because of his knowledge of the subject, his own welcome contributions to the book and also because he came to the symposium in Melbourne from London, but had spent much of his life in Australia and has now returned there, to continue his research, at the Hall Institute.

The Director of the Foundation was assisted at the conference by Miss Ruth Porter, and Mrs. Nancy Spufford gave her customary expert administrative help and guidance from a distance.

The meeting was international and the members from different countries joined in making it not only a sincere personal tribute to Sir Macfarlane Burnet, but also an expression of world recognition of his unique contributions to medical research. Every reader who profits by and enjoys reading these papers and discussions will be joining to some extent in this grateful tribute, and it is certain that nothing can give more pleasure to Sir Macfarlane and to ourselves, than if some original research, in some part of the world, were to be promoted by a reading of this book.

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G. J. V. Nossal	Walter and Eliza Hall Institute of Medical Research, Melbourne
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N. F. Stanley	Dept. of Microbiology, University of Western Australia, Perth
J. Šterzl	Dept. of Immunology, Institute of Microbiology, Czechoslovak Academy of Science, Prague
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M. F. A. Woodruff	Dept. of Surgical Science, University of Edinburgh

* Not present at the meeting.

CHAIRMAN'S OPENING REMARKS

Sir Macfarlane Burnet

It has given us all in Melbourne very great pleasure that the Ciba Foundation has found it possible to mount this symposium here, and for me it is of course a very special source of gratification. My colleagues at the Institute have spared no effort to make the meeting a success and to see that our visitors take away a happy memory of Melbourne.

The outstanding feature of the development of immunology in the last ten years has been the recognition of the function of the lymphocyte and of the importance of the thymus in the immune process. The two aspects have developed together but the connexion between them is still a matter rather for speculation than for experiment. In symposia like these, one looks more for the impact of different points of view and for suggestions of new lines of work to be followed in the future than for the presentation of exciting new experimental results. We all tend to get caught within the interests and ideas of our own group of associates and it is very good if we can be gently jolted, for a time at least, into another orbit.

To me the central feature of our discussions in the next three days will be the population dynamics of thymus cells, their origin and entry, their changes within the thymus and their disappearance from it. What happens to all the cells produced in the thymus? This is a question that has never been satisfactorily approached at the experimental level. Both for the mammalian thymus and the avian bursa new technical approaches are badly needed. Until they are forthcoming I am almost embarrassed by the number of in-

teresting but sometimes mutually incompatible functions one can ascribe to the thymus in armchair theorizing.

There may be surprises in store for us. In my own contribution, for instance, I am going to talk about a phenomenon which, histologically speaking, is one of the most striking in experimental pathology but which does not fit into any pattern of thinking about the thymus.

In Otago, and in my own laboratory, we have been strongly influenced by what happens in the New Zealand black (NZB) mice and their hybrids. In our clinical research unit here, at the Johns Hopkins Hospital, in Edinburgh, and in Glasgow the pathology of the human thymus is a matter of keen interest. Somehow the pathological manifestations must be brought into relation with our experimental findings and the pattern which is emerging from them.

I hope that within the next few months we shall see real advances that have been sparked off by this symposium on the thymus.

CYTOLOGICAL EVIDENCES OF SECRETION IN THE THYMUS

SAM L. CLARK, JR.†

Department of Anatomy, Washington University School of Medicine, St. Louis, Missouri

EVIDENCE is accumulating that the thymus secretes a hormone. The crippling effects of early thymectomy upon development of the lymphoid system and of immunological competence can be reversed by a thymic graft, even when that graft is enclosed in a diffusion chamber that precludes any exchange of cells between graft and host (Levey, Trainin and Law, 1963; Law *et al.*, 1964; Osoba and Miller, 1964). There are several reports that saline extracts of thymus will induce proliferation of lymphocytes. Metcalf (1956) recorded increased numbers of circulating lymphocytes in newborn mice and thymectomized adults injected with a heat-labile extract of mouse thymus; Gregoire and Duchateau (1956) described hyperplasia of lymphoid tissues in adult rats treated with extracts from rabbit and pig thymus; Camblin and Bridges (1964) used extracts of rat or rabbit thymus to restore circulating levels of lymphocytes to normal in adult rats rendered lymphopenic by irradiation; and Klein, Goldstein and White (1965) detected increased rates of incorporation of radioactive precursors into DNA and protein in lymph nodes of mice injected with thymic extracts from mice, rats and calves. Acceler-

† Career Development Awardee of the National Institutes of Health, United States Public Health Service

ated synthesis of DNA was observed within an hour of injection—an indication that the stimulus to proliferation may act at this level. Extracts of other lymphoid organs and foreign proteins, used as controls in these studies, were ineffective. Thus the thymus appears to contain some heat-labile, water-soluble material that rapidly induces proliferation of lymphocytes in other lymphoid organs. Under the bizarre conditions in diffusion chambers, the thymus seems to release, as well as to contain, such a factor.

There are indications that this material is associated with the epithelial cells of the thymic medulla. Metcalf (1956) reported that extracts of medulla were more effective than extracts of cortex. Osoba and Miller (1964) stated that within diffusion chambers thymic lymphocytes soon degenerated leaving a gland which, although consisting almost entirely of epithelial cells, was still capable of overcoming the effects of thymectomy. Gregoire and Duchateau (1956) obtained their extracts from thymuses in which an almost purely epithelial structure had been produced by destroying the lymphocytes with irradiation.

Thymus medullary epithelial cells resemble glandular cells. In the first place they are truly epithelial. This has been stated by some early histologists and confirmed by electron microscopy (Clark, 1963; Hoshino, 1963; Weiss, 1963). In the cortex the epithelial cells extend as narrow septa between infiltrating lymphocytes and possess few cytoplasmic organelles, but in the medulla, where lymphocytes are scarce, the large epithelial cells form cords and islands, possess many of the cytological features of secretory cells and, with advancing age, produce cysts and cystic foci of degeneration—Hassal's corpuscles (Arnesen, 1958; Kohnen and Weiss, 1964; Izard, 1965).

There are three types of cytoplasmic inclusions in medullary epithelial cells that might be considered to be secretory products. Most obvious are large collections of amorphous material that stain with the periodic acid-Schiff (PAS) technique for poly-

saccharides. These collections occur both intracellularly, and in extracellular spaces that may be lined by cilia (Hoshina, 1963). Expansion of such extracellular collections produces the epithelial cysts that accumulate with age. Second, there are clusters of vacuoles containing an amorphous material that gives them the appearance of mucoid droplets (Clark, 1963) like those in the mucous cells of the thymus in lower vertebrates (Bargmann, 1943). Finally, there are small dense granules that might be considered secretory (Clark, 1963).

In order to obtain functional evidence for secretory activity, to confirm or refute the structural evidence, mice were injected with radioactive sulphate, glucosamine or leucine, and sites of synthesis and storage of polysaccharides and proteins were identified within the thymus by radioautography.

RADIOAUTOGRAPHIC EXPERIMENTS

Inbred Swiss or leaden mice of both sexes were injected intraperitoneally with carrier-free $Na_2{}^{35}SO_4$ (Oak Ridge National Laboratories, Oak Ridge, Tennessee and New England Nuclear Corporation, Boston), [^{14}C]glucosamine or tritium-labelled leucine (both from the New England Nuclear Corporation). At intervals from 15 minutes to 48 hours later, the mice were killed and the thymus prepared for radioautography and electron microscopy, utilizing fixation in buffered osmium tetroxide (Clark, 1963), glutaraldehyde (Sabatini, Bensch and Barrnett, 1963), or both, and butyl methacrylate or epoxy resins (Lockwood, 1964) for imbedding. Radioautographs were prepared by the techniques of Caro and Tubergen (1962), exposed for 1 to 21 weeks, and stained with hot 0·5 per cent azure B bromide for light microscopy or uranyl acetate for electron microscopy. Electron micrographs were obtained with an RCA EMU 3C electron microscope. Protocols of the experiments are summarized in Table I.

TABLE I

RADIOAUTOGRAPHIC EXPERIMENTS

Isotope	*Dose (microcuries)*	*Number of animals*	*Age (months)*	*Time interval (hours)*
$Na_2{}^{35}SO_4$	300	3	$\frac{1}{2}$	1
	300	3	$\frac{1}{2}$	4
	120	2	1	3
	200	2	6–9	3
	200	1	6–9	24
[^{14}C]glucosamine	20	5	1	3
[^{3}H]leucine	125	1	6–9	$\frac{1}{4}$
	125	1	6–9	3
	200	1	6–9	$\frac{1}{6}$
	200	1	6–9	$\frac{1}{2}$
	200	1	6–9	24
	200	1	6–9	48

PLATE I, FIGS. 1–5

FIG. 1. Radioautograph of thymus from a 15-day-old mouse killed 1 hour after intraperitoneal injection of 300 μc of carrier-free $Na_2{}^{35}SO_4$; radioautograph exposed for 48 days. The medulla (the pale central region) contains several foci of silver grains. There is light radioautographic label over the rest of the medulla, but the cortex (in opposite corners of the picture) contains little label. (× 275).

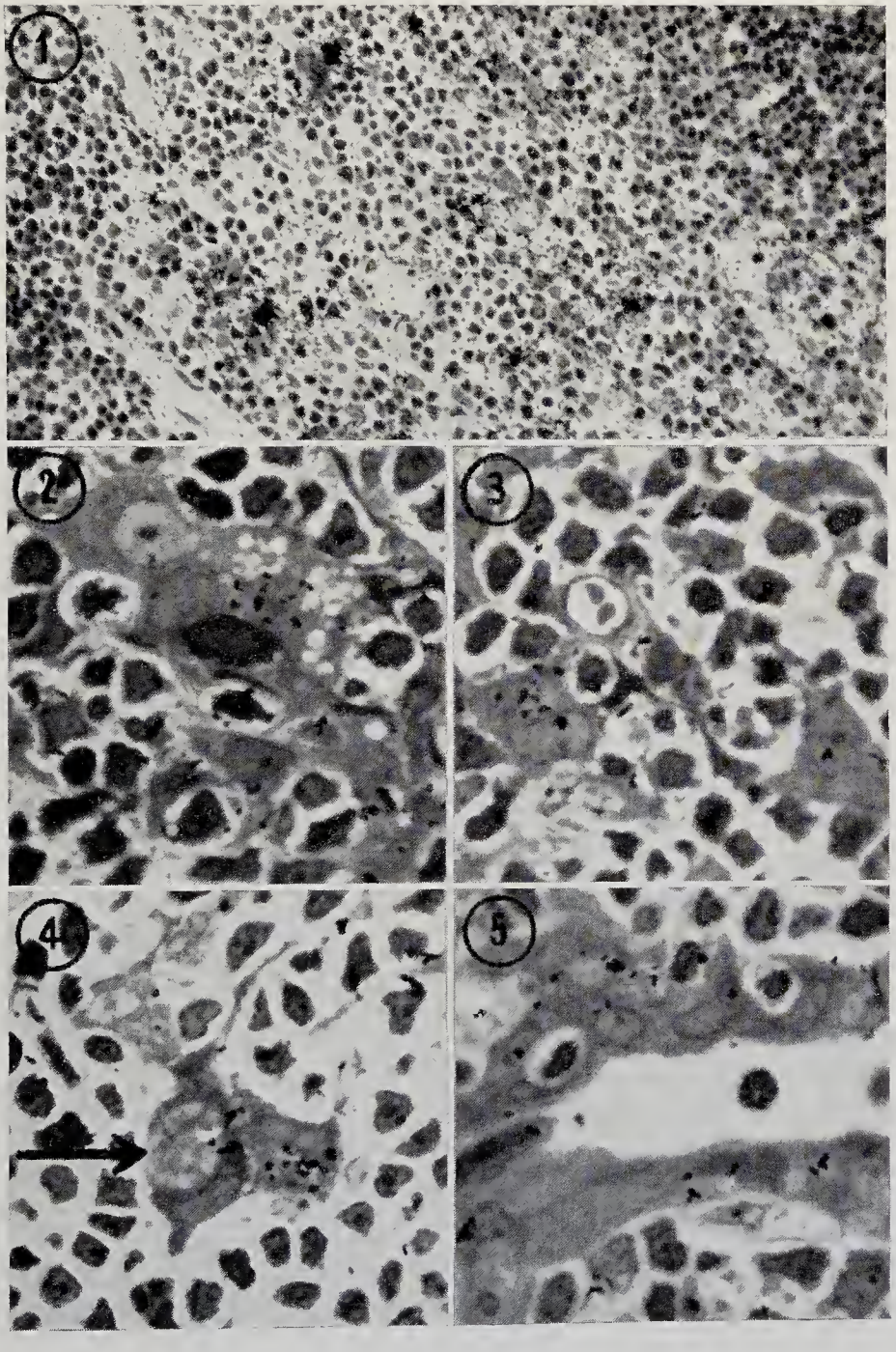
FIGS. 2–5. Radioautographs of thymic medulla from an adult mouse killed 3 hours after intraperitoneal injection of 200 μc of $Na_2{}^{35}SO_4$. Tissue embedded in methacrylate and radioautographs exposed for 15 weeks. (× 1000).

FIG. 2. Nest of medullary epithelial cells with clusters of silver grains over the cytoplasm. The label lies near the nucleus in region usually occupied by the Golgi apparatus. Surrounding the labelled area are numerous clear vacuoles free of label. Infiltrating lymphocytes are also unlabelled.

FIG. 3. Epithelial cell on the left contains a collection of silver grains over what appears to be a cytoplasmic inclusion or large vacuole.

FIG. 4. Right-hand epithelial cell in this pair is labelled over the cytoplasm near the nucleus. The left-hand cell surrounds a small ciliated cyst (unlabelled) whose contents are moderately dense (arrow).

FIG. 5. Large cyst of epithelial cells surrounds lumen containing little but cellular debris. Cytoplasm of some of the surrounding epithelial cells is labelled. In the upper left corner label lies over clustered vacuoles.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RADIOACTIVE SULPHATE

One to 4 hours after injection, radioautographic labelling was confined almost entirely to perivascular and interlobular connective tissue and to discrete foci within the thymic medulla (Plate I, Fig. 1). The connective tissue label appeared to be largely extracellular—presumably it represented sulphated mucopolysaccharides of the ground substance. The medullary foci lay over the cytoplasm of epithelial cells. In lightly labelled preparations, silver grains could be localized in the region near the nucleus usually occupied by the Golgi apparatus (Plate I, Figs. 2 and 4), over clustered vacuoles (Plate I, Fig. 5), and over large cytoplasmic inclusions (Plate I, Fig. 3). At 1 and 3 hours after injection most of the large cytoplasmic inclusions and ciliated epithelial cysts remained unlabelled (Plate I, Fig. 4), even though there was heavy label in the adjacent cytoplasm, but at 4 hours some of these structures were labelled (Plate II, Fig. 8). Electron microscopic radioautography confirmed the presence of label over clustered vacuoles and the surrounding cytoplasm in medullary epithelial cells (Plate II, Figs. 6 and 7). A few silver grains were found over lymphocytes and macrophages. No specific association of silver grains with the small dense granules in epithelial cells could be detected, but this is not surprising in view of the fact that the granules are smaller than the limits of resolution of radioautography.

In the animal killed 24 hours after an injection of radioactive sulphate, the label was approximately as heavy as it had been at earlier intervals, but it was spread diffusely throughout the thymus and showed no preference for medullary epithelial cells.

The intensity of labelling was more or less similar at all time intervals, but the greatest number of labelled epithelial cells was found in the youngest animals examined—those 15–17 days old. Although medullary epithelial cells were heavily labelled, they were not labelled as heavily as goblet cells in the small intestine in the same animals.

RADIOACTIVE GLUCOSAMINE

Three hours after an injection of radioactive glucosamine radioautographic labelling was lighter and more diffuse than that seen after injections of radioactive sulphate, but there were definite discrete foci of labelling over the juxtanuclear cytoplasm and over clustered vacuoles of medullary epithelial cells (Plate II, Fig. 9).

RADIOACTIVE LEUCINE

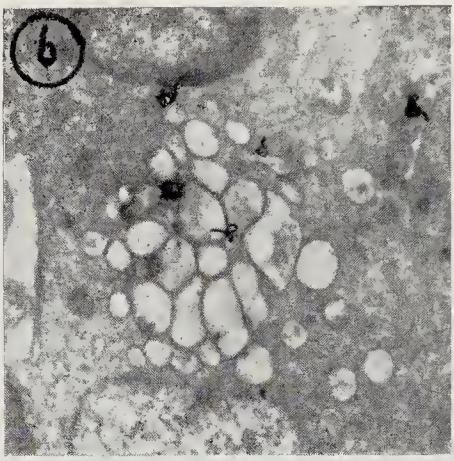
At all intervals from 15 minutes to 48 hours after injection considerable label was present but it was scattered diffusely throughout the thymus. The most definite foci of labelling were large lymphocytes in the cortex. Medullary epithelial cells were labelled over both nucleus and cytoplasm, but the label was neither heavy nor discretely localized.

HISTOCHEMISTRY

A random collection of thymuses from mice of various ages, prepared over a period of 4 years by several different techniques of fixing and imbedding, were cut in sections 2 μ thick, stained with the PAS technique and counterstained either with Bismark brown or azure B bromide. Sections imbedded in methacrylate, from which the plastic had been removed by soaking in xylol, stained more intensely than sections imbedded in epoxy resins and also could be stained with alcian blue (3 per cent in 70 per cent ethanol), but cytological preservation was poorer than after embedding in epoxy resins.

ADULT MICE

Medullary epithelial cells contained numerous examples of the three types of cytoplasmic inclusions described in the introduction. Most were PAS-positive, but many were basophilic and a few stained with alcian blue (Plate II, Fig. 10).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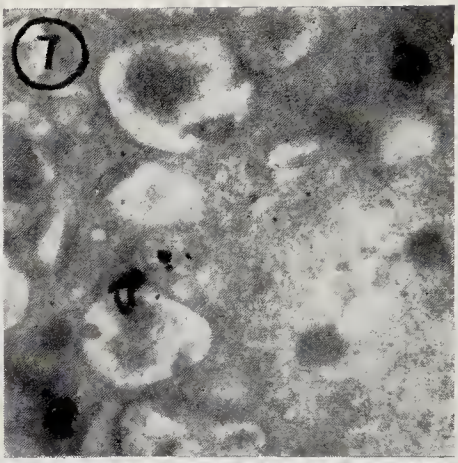
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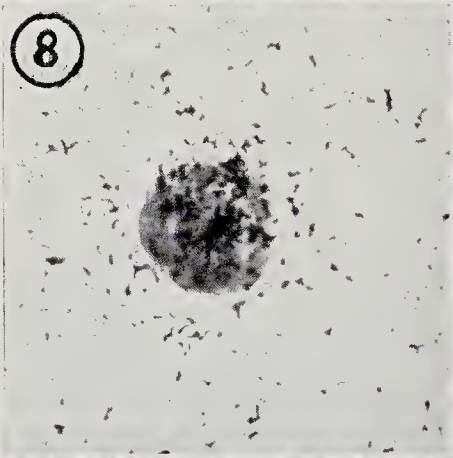
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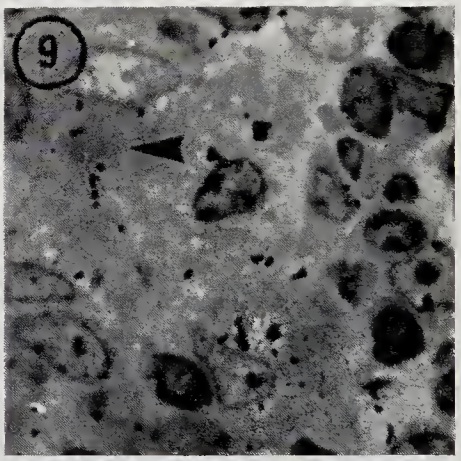
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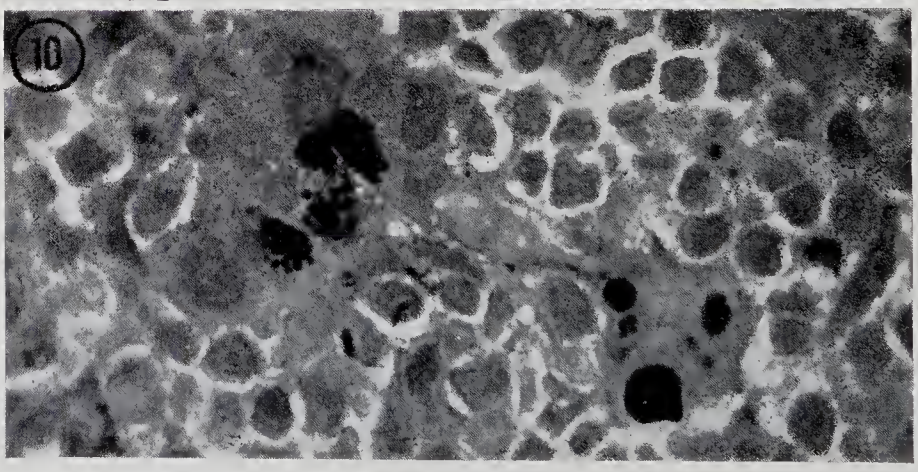
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1. The small dense granules varied continuously in size from the limit of resolution of the light microscope (about 0·25 μ) up to several microns in diameter.

2. Clustered vacuoles usually occupied the region of the cytoplasm near the nucleus, but small clusters also occurred along some of the attenuated cytoplasmic extensions in both cortical and medullary epithelial cells, (Hoshino, 1963). Some clusters appeared empty, others contained an amorphous material, and still others contained small dense granules similar to those described above.

3. Large dense inclusions of amorphous material, ranging in diameter from about 10 μ upward, occupied both intracellular

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PLATE II, FIGS. 6–10

FIGS. 6 and 7. Electron microscopic radioautographs prepared from the same thymus as shown in Fig. 1, 1 hour after injection of $Na_2{}^{35}SO_4$; radioautographs exposed for 12 weeks. In both pictures curly silver grains lie over clustered vacuoles in the cytoplasm of medullary epithelial cells, as well as over the surrounding cytoplasm. (Fig. 6, × 8000; Fig. 7, × 15,500).

FIG. 8. Radioautograph of thymic medulla from 15-day-old mouse killed 4 hours after intraperitoneal injection of 300 μc of $Na_2{}^{35}SO_4$; radioautograph exposed for 48 days. Note dense cluster of silver grains over basophilic contents of epithelial cyst in the centre of the picture. There is lighter label over the surrounding medulla, but cellular details are not visible because the picture was under-exposed to show up silver grains over the dense cystic contents. (× 1000).

FIG. 9. Radioautograph of thymic medulla from 1-month-old mouse killed 3 hours after intraperitoneal injection of 20 μc of [^{14}C]glucosamine; radioautograph exposed for 21 weeks. Note epithelial cell with labelled vacuoles in its cytoplasm in lower part of picture and several silver grains over what appears to be a large cytoplasmic inclusion in an epithelial cell in upper left-hand corner of picture (arrow). (× 1000).

FIG. 10. Thymic medulla from an adult mouse, about 6 months old, embedded in butyl methacrylate and stained sequentially with alcian blue, PAS, and Bismark brown. Note clusters of PAS-positive cytoplasmic droplets, corresponding to the clustered vacuoles in Fig. 12, in nest of epithelial cells in upper left corner. Cells towards lower right part of field contain several large dense inclusions and a few droplets—all PAS-positive. The rest of the field is stained with the basic dye, Bismark brown. (× 1000).

and extracellular positions. Some intracellular inclusions had the appearance of clustered vacuoles densely filled with amorphous material. Extracellular accumulations appeared to form a continuum with the larger epithelial cysts that increase in number with advancing age (Arnesen, 1958). Some inclusions stained both with PAS and with alcian blue or a basic dye, either as a homogeneous mixture of colours, or in a variegated fashion.

These observations confirm those of Ito (1959), Hoshino (1963) and Izard (1965), who noted, also, that some of the large inclusions stained with aldehyde fuchsin and for alkaline phosphatase.

CHANGES WITH AGE

The prevalence and staining characteristics of the three types of cytoplasmic inclusions were compared in a series of sections taken from mice of various ages; all the sections were fixed with osmium tetroxide, imbedded in epoxy resins and stained with PAS and azure B bromide. This comparison is summarized in Table II and examples are depicted in Plate III, Figs. 11–14.

From this incomplete series of observations it appears that the thymic medulla began to develop late in gestation as islands of epithelial cells containing small basophilic granules (Plate III, Fig. 11). Even at this early stage there were a few incipient Hassal's corpuscles—concentric nests of epithelial cells surrounding a cystic lumen that contained amorphous basophilic material. By the end of the first postnatal week clustered vacuoles had become prominent and there were a few large inclusions (Plate III, Fig. 12). Some were PAS-positive, but most were basophilic. The medulla had grown to become a continuous but still relatively small part of the thymus. By the beginning of the third week after birth the medulla had reached adult proportions and contained many large inclusions, most of which now stained with PAS. Clustered vacuoles and basophilic granules were still numerous, and at this age there appeared to be more epithelial

TABLE II

CYTOLOGY AND HISTOCHEMISTRY OF THYMIC MEDULLA

		Prevalence of epithelial inclusions					
		Small granules (1).		*Clustered vacuoles* (2).		*Large inclusions* (3).	
Age	*Number of animals*	*Basophilic*	*PAS+*	*Basophilic*	*PAS+*	*Basophilic*	*PAS+*
11½-day embryo	4	0	0	0	0	0	0
14½-day embryo	2	0	±	0	0	0	0
Full-term embryo	2	+++	±	0	0	0	0
5 days	2	+++	+	+++	+	+	+
7 days	2	++	+	++++	+	+	+
15–17 days	5	++	+	++++	++	+	+++
31 days	7	+	+	+	++	+	++
6–9 months	3	+	+	+	++	+	++

cells incorporating radioactive sulphate than in older age groups. With advancing maturity many of the medullary epithelial cells no longer contained inclusions and increasing numbers of lymphocytes infiltrated the medulla, reducing the apparent concentrations of granules, vacuoles and large inclusions. In addition, the small granules decreased disproportionately and were scarce in adult animals.

The pale outer region of the cortex, consisting of epithelial cells and large lymphocytes, appeared late in foetal life, was still present a week after birth but had almost disappeared by two weeks after birth. It showed little evidence of secretion.

Thus the three types of epithelial inclusions become prominent at different ages. Small granules were numerous before birth and decreased in number 2 to 3 weeks after birth. Clustered vacuoles burgeoned during the first postnatal week, but large inclusions

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PLATE III, FIGS. 11–14

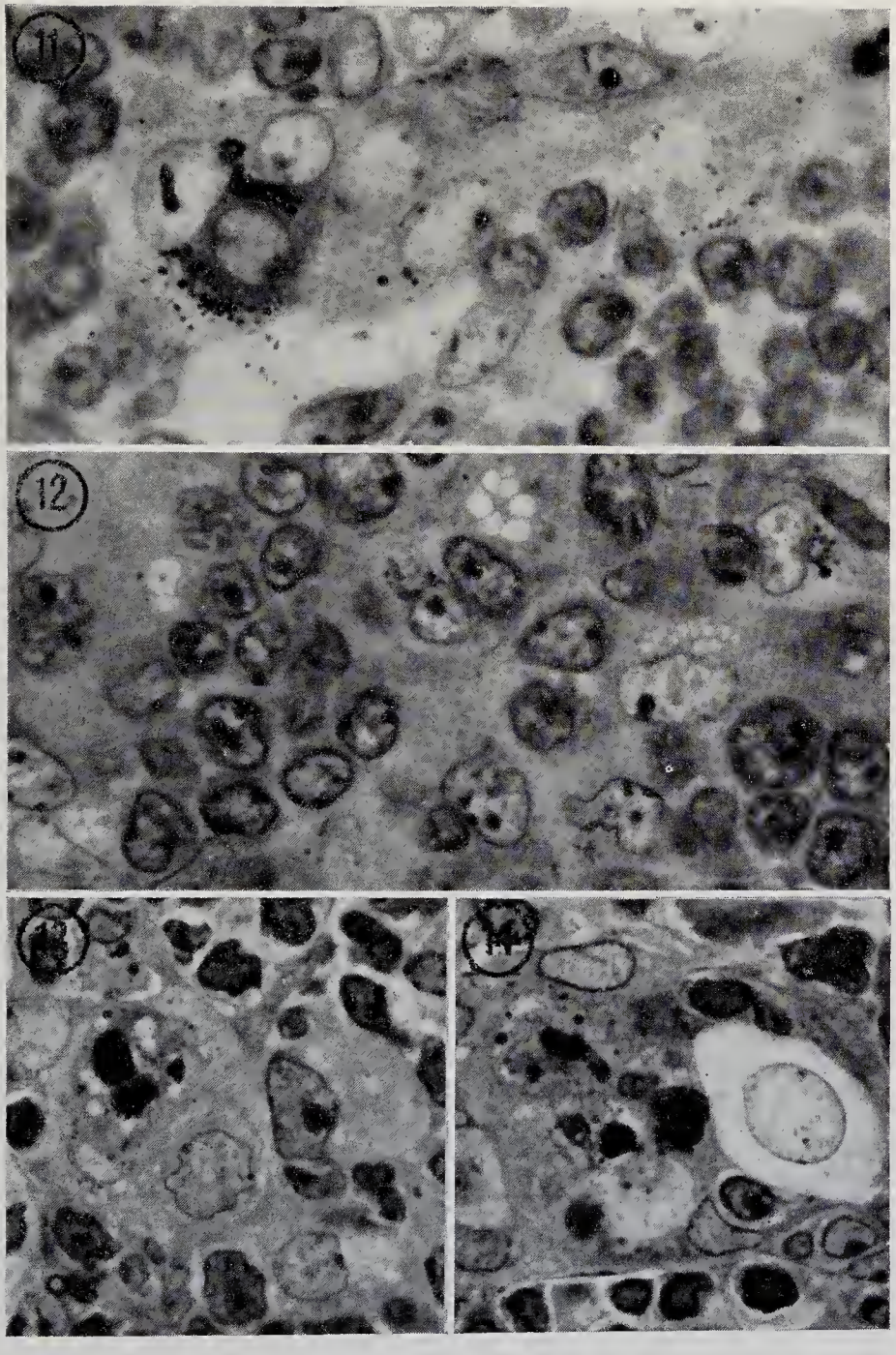
FIG. 11. Island of epithelial cells from thymus of a full-term foetus (mouse), stained with PAS and azure B bromide. Note, on the left, small basophilic granules scattered through cytoplasm of the epithelial cells and concentrated in cell with most basophilic cytoplasm to the left of the picture; on the right, a cluster of cytoplasmic vacuoles. Densely packed lymphocytes surround the island of epithelial cells. Such islands serve as a thymic medulla before birth. (× 1000).

FIG. 12. Part of rudimentary thymic medulla from a 7-day-old mouse, stained with PAS and azure B bromide. Note 4 clusters of vacuoles and 2 groups of basophilic granules in cytoplasm of epithelial cells. The cells with darker nuclei are infiltrating lymphocytes. (× 1000).

FIGS. 13 and 14. Thymic medulla from a 31-day-old mouse, stained with PAS and azure B bromide. (× 1000).

FIG. 13. Nest of medullary epithelial cells containing 2 large basophilic cytoplasmic inclusions surrounded by clear vacuoles. Note smaller vacuoles in other epithelial cells.

FIG. 14. Nest of epithelial cells containing several large dense cytoplasmic inclusions, vacuoles, and small basophilic granules. The lowest inclusion is PAS-positive, the rest are basophilic. The very pale epithelial cell appears to be degenerating. Such nests containing large inclusions and degenerating epithelial cells seem to be the precursors of Hassal's corpuscles.


11
12
13
14

did not become prominent until two weeks after birth. All three types of inclusions were predominantly basophilic until the second or third week after birth, when an increasing proportion become PAS-positive. Arnesen (1958) has reported that large inclusions and epithelial cysts increase further with advancing age. Although they may resemble thyroid follicles they do not incorporate radioactive iodine (Arnesen and Kierulf, 1961).

ELECTRON MICROSCOPY

The high resolution of the electron microscope afforded a more detailed view, not only of the three types of epithelial inclusions examples of which have been reported already (Clark, 1963; Hoshino, 1963; Kohnen and Weiss, 1964; Izard, 1965), but also of the structure of the rest of the epithelial cell. It is this context in which the inclusions lie that furnishes the best morphological evidence for their being secretory. Epithelial nuclei were large and pale, with the dispersed chromatin and prominent nucleoli characteristic of cells actively synthesizing RNA, that is, cells either growing and differentiating, or secreting (Plate V, Fig. 16). The cytoplasm contained ergastoplasm, or rough-surfaced endoplasmic reticulum, which is characteristic of cells manufacturing protein for secretion rather than for internal use (Plates IV and VI, Figs. 15, 17 and 18), and the Golgi apparatus was well developed as it is in cells that secrete proteinaceous or mucoid materials (Plates IV, VI and VII, Figs. 15, 17 and 19) (Caro and

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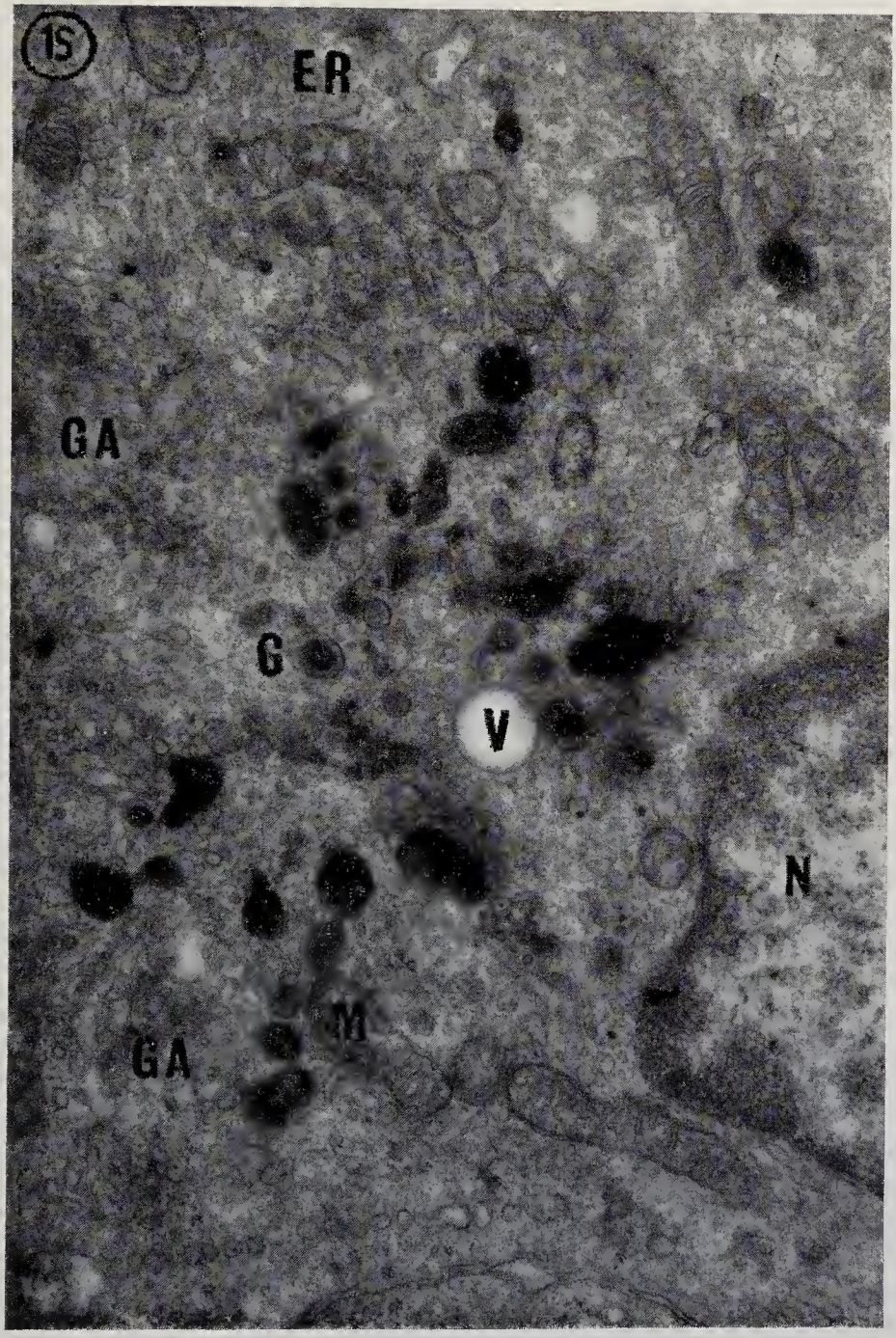
PLATE IV, FIG. 15

FIG. 15. Electron micrograph from a 5-day-old mouse. Near the nucleus (N) of an epithelial cell is a cluster of small dense granules of the type characteristic of late foetal and early postnatal life (Fig. 11). Each granule is surrounded by a smooth membrane. Note, in the centre of the cluster, a vacuole (V), small vesicles with amorphous contents of low density, and rudimentary granules of intermediate density (G). Surrounding this focus of granules are smooth-surfaced elements of the Golgi apparatus (GA), rough-surfaced endoplasmic reticulum or ergastoplasm (ER), and mitochondria (M). (× 25,000).


15
ER
GA
G
V
N
M
GA

Palade, 1964; Lane *et al.*, 1964). Absorptive cells also contain well-developed Golgi complexes, but they lack the constellation of other features described above. Thus the medullary epithelial cells possessed the machinery for secretion. Furthermore, the three types of inclusions lay in an appropriate relationship to this machinery.

1. The small dense granules, each enclosed in a single membrane as are other secretory granules, usually lay clustered in the region of the Golgi apparatus, with dense, well-formed granules located peripherally and less dense, rudimentary granules in the centre (Plate IV, Fig. 15). Similar arrangements have been observed in other cells where a secretory product is concentrated into granules in the Golgi region (Caro and Palade, 1964).

2. The clustered vacuoles possessed smooth membranes with irregular microvilli extending into their lumen. Characteristically the vacuoles lay in the Golgi region but also had close relationships with ergastoplasm (Plates V, VI and VII, Figs. 16 to 20). In these respects they resembled mucous droplets forming within vacuoles in the Golgi region of intestinal goblet cells (Godman and Lane, 1964). Their contents varied both in type and concentration. Most vacuoles contained an amorphous material, either uniformly dispersed (Plates V, VII, Figs. 16 and 20) or clumped (Plate VI, Fig. 18). Some also enclosed small dense granules similar in appearance to those described above (Plate VI, Fig. 17), and a few contained a membranous material. A few clustered vacuoles were

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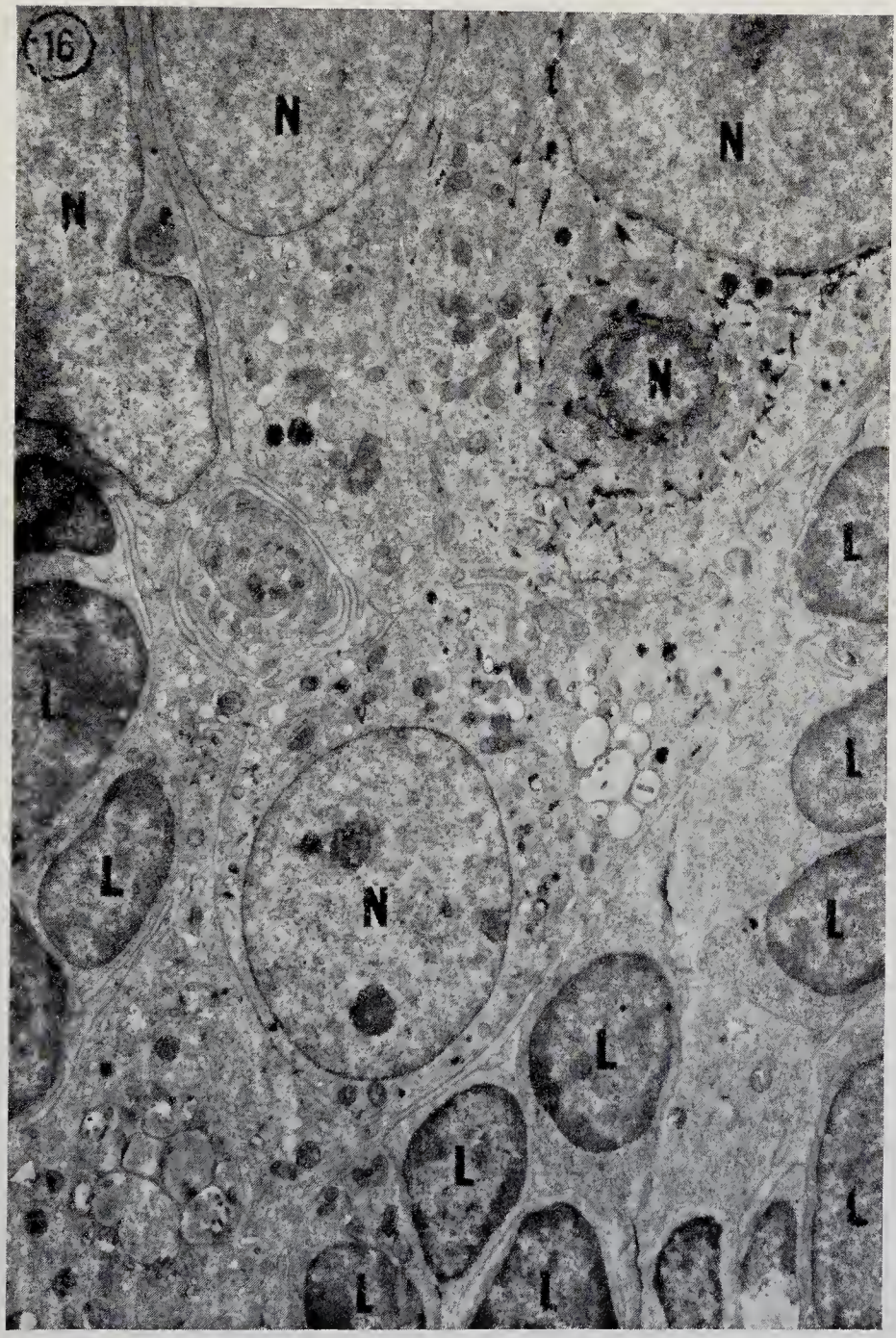
PLATE V, FIG. 16

FIG. 16. Electron micrograph from a 5-day-old mouse. Nest of epithelial cells surrounded by infiltrating lymphocytes (L) resembles the collections of vacuolated epithelial cells in Fig. 12. The epithelial nuclei (N) are large, with dispersed chromatin and prominent nucleoli characteristic of cells actively engaged in the synthesis of ribonucleoprotein. Cell in the centre of the field contains a cluster of vacuoles with intruding microvilli as well as small dense granules. Note another cluster of vacuoles with denser amorphous contents in lower left corner and cells containing tonofibrils in upper right corner. (× 5000).



THY.—2

surrounded by the basal bodies of cilia or by complete cilia (Plate VII, Fig. 19). Although the space within the vacuoles might have proved to be extracellular in another plane of section, it seems possible that these cilia were entirely intracellular (Hoshino, 1963).

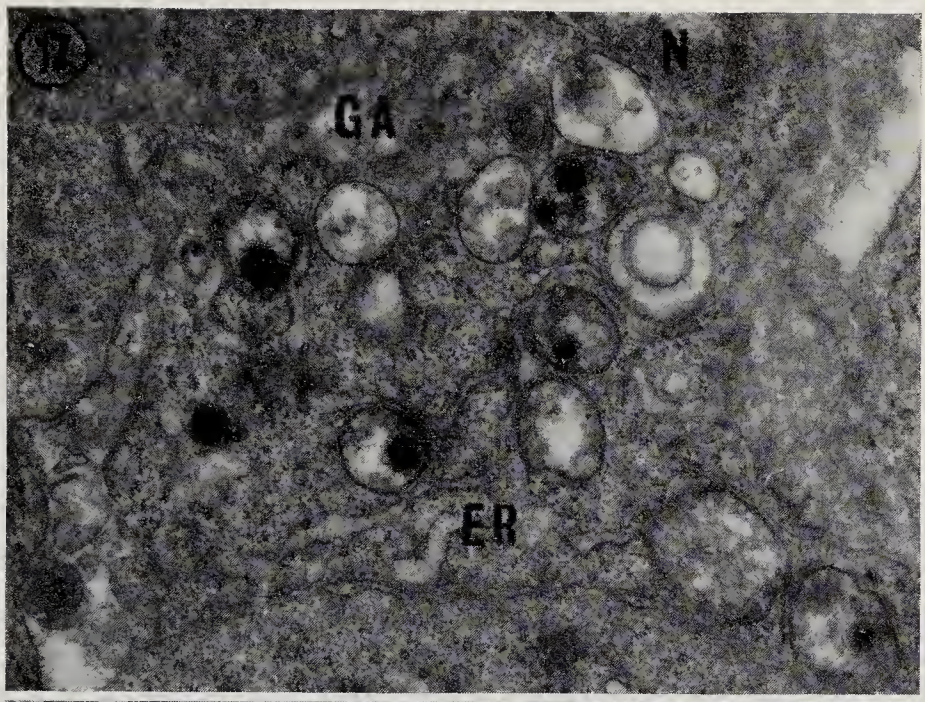
3. The large inclusions seemed to be of at least two types. One had the appearance of a cluster of vacuoles in which the accumulated amorphous material had reached such a concentration that it would be dense when viewed in the light microscope after staining with PAS (Plate VII, Fig. 20). The other type was a single, rather than multilocular, accumulation of amorphous material within a space bounded by microvilli or cilia (Plate VII, Fig. 21). In many cases this type of inclusion was demonstrably extracellular and surrounded by several epithelial cells held together at their junctions by desmosomes or terminal bars. This second type of large inclusion had the appearance of a blind glandular acinus or follicle filled with secretory material.

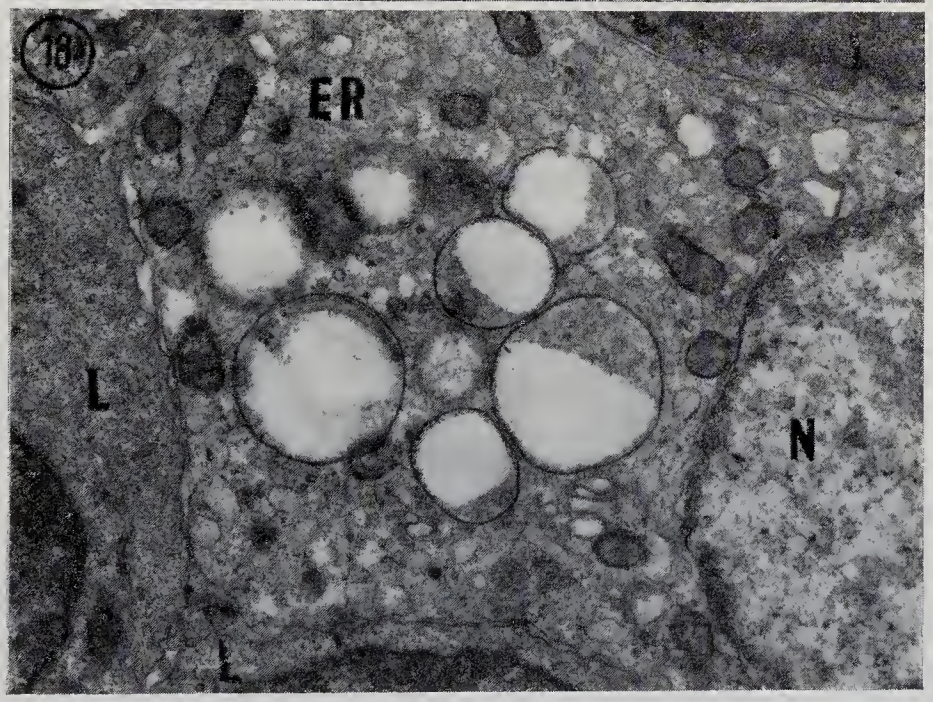
The bodies of Foa-Kurloff are large, PAS-positive inclusions that arise at the corticomedullary border in guinea pigs during acute involution of the thymus. They contain myelin figures that set them apart from the large inclusions seen in mice and the cell in which they occur has not been identified (Izard, Bimes and Guilhem, 1964). Their relationship to the present discussion is not clear.

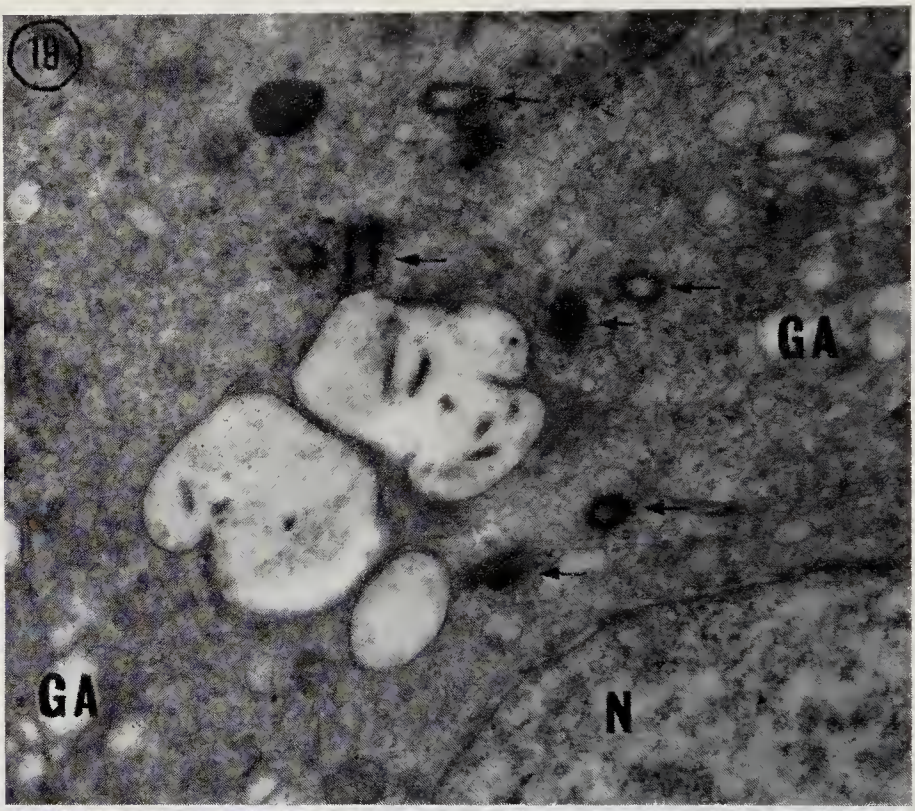
PLATE VI, FIGS. 17 and 18

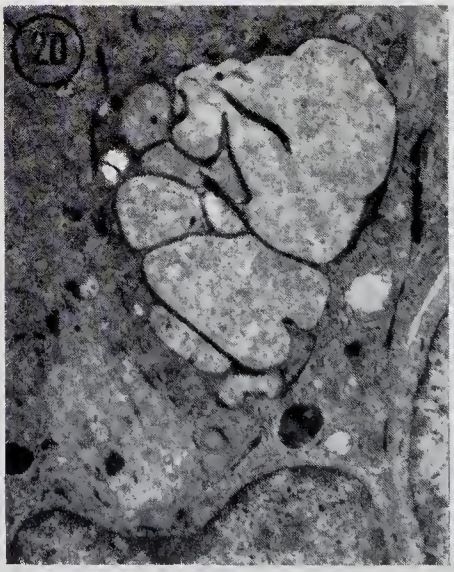
FIG. 17. Electron micrograph from a 5-day-old mouse. In the region of the Golgi apparatus (GA), near the nucleus (N) of this epithelial cell, note collection of vacuoles containing membranous material, amorphous material and small dense granules (see also Fig. 15). These structures lie in close relationship to both the Golgi apparatus and membranes of the ergastoplasm (ER). (× 21,500).

FIG. 18. Electron micrograph from a 5-day-old mouse. Note cluster of vacuoles with unevenly distributed amorphous contents surrounded by mitochondria and ergastoplasm (ER) near nucleus (N) of an epithelial cell. L = lymphocyte. (× 15,500).


17
N
GA
ER


18
ER
L
N
L


19
GA
GA
N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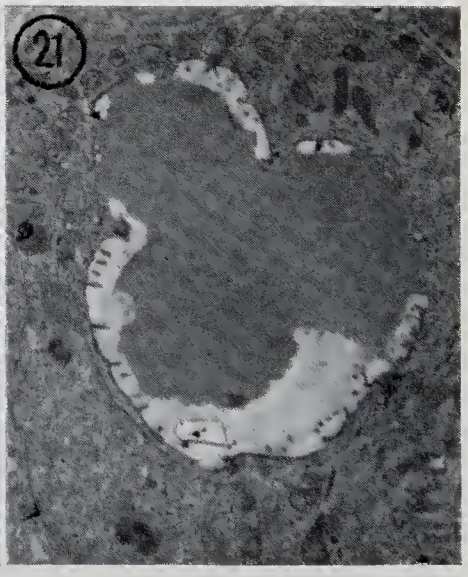
21

SIALIC ACID ASSAYS

In an attempt to obtain a quantitative measure of the polysaccharides presumed to be constituents of the epithelial inclusions, Warren's (1959) method for determining sialic acid in tissues was applied to the thymuses and submaxillary glands of five young adult buffalo rats. The assay was complicated by the high levels of nucleic acid in the thymus, but after extraction with isoamyl alcohol levels of sialic acid were measured ranging from 23 to 58 μmole of N-acetyl neuraminic acid equivalents per 100 g. of tissue. These values were much lower than those found in submaxillary gland—310–471 μmole per 100 g.—and were near the lower limits of sensitivity of the method. Therefore, it seemed impractical to pursue this method as a means for measuring functional fluctuations in thymic secretion. Perhaps counting the radioactivity of incorporated sulphate would serve the purpose.

DISCUSSION

What, then, is the evidence that any or all of the inclusions seen in medullary epithelial cells are secretion products? There is circumstantial evidence in the location of these inclusions in

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PLATE VII, FIGS. 19–21

FIG. 19. Electron micrograph from a 5-day-old mouse. Note, near nucleus (N) of an epithelial cell, and between elements of the Golgi apparatus (GA), a cluster of vacuoles surrounded by centrioles (arrows) that may be the basal bodies of cilia. One such putative basal body abuts directly on a vacuole. (× 19,000).

FIG. 20. Electron micrograph of medullary epithelial cell from an adult mouse. Note, in the cytoplasm, a large cluster of vacuoles containing a moderate concentration of amorphous material. This is one form of the large PAS-positive inclusions found with increasing frequency in older animals. Note also the delicate filaments in the surrounding cytoplasm. (× 8000).

FIG. 21. Electron micrograph of epithelial cyst from thymic medulla of an adult mouse. Note concentrically arranged epithelial cells surrounding a large intercellular space incompletely filled by amorphous material and lined with microvilli. This structure resembles the cyst in Fig. 8. (× 4500).

epithelial cells equipped with the characteristic machinery of secretion. Radioautography provides direct evidence that these cells are active in the synthesis of some sulphated mucopolysaccharide. The rapid incorporation of sulphate and glucosamine is typical of cells that secrete a mucoid product (Peterson and Leblond, 1964; Godman and Lane, 1964; Lane *et al.*, 1964), whereas the slow incorporation of leucine seems inconsistent with secretion of a product that is chiefly protein (Warshawsky, Leblond and Droz, 1963; Caro and Palade, 1964).

For a product to be called secretory it must be released from the cell in which it is made in order to act at a distance. Two lines of evidence suggest that this is true for the thymus. First, some of the basophilic or PAS-positive material within the lumens of epithelial cysts became labelled 4 hours after an injection of radioactive sulphate, as if material synthesized by the epithelial cells had been excreted into the cysts. Second, the dispersion of label seen in one animal 24 hours after injection of radioactive sulphate, if confirmed by repeated experiments, would indicate a complete turnover of sulphated material within less than 24 hours.

If these experiments do indicate the origin of a thymic hormone, they also provide hints on its chemical nature that may be useful in guiding efforts to extract and purify it. Staining with PAS and the incorporation of glucosamine both point to its polysaccharide nature, while staining with alcian blue, basophilia and the incorporation of sulphate indicate that it is a sulphated acid-mucopolysaccharide. It has been reported that the inclusions contain alkaline phosphatase (Ito, 1959; Smith, 1961).

If we accept that medullary epithelial cells manufacture and secrete an acid mucopolysaccharide, it is possible, on the basis of the cytological and histochemical evidence, to reconstruct a hypothetical natural history of thymic secretion in the mouse.

As to the onset of secretion, clustered vacuoles become prominent during the first week after birth. This schedule of development is consistent with Metcalf's (1956) report that the lymphocytosis

stimulating factor was undetectable in the thymus of mice 2 days old, but had reached high levels by 7 days after birth. However, lymphocytopoiesis is active in the thymus before birth, at a time when clustered vacuoles are few but basophilic granules are numerous. Perhaps these granules are the first signs of thymic secretion. The increase in clustered vacuoles after birth could be taken as evidence of an accelerated rate of secretion that does not allow time for the product to be concentrated in granules. According to this interpretation, vacuoles containing small dense granules would be in a transitional state between the two types of secretion.

The large inclusions that become so numerous two weeks after birth appear to develop from the progressive accumulation of amorphous material within clustered vacuoles and cystic extracellular spaces. If this represents a shift from rapid release to storage of the secretory product, it may indicate some moderation of secretory activity. The change in staining characteristics of the inclusions from basophilic to PAS-positive that occurs as the mouse matures may indicate some change in the chemical nature of the secretory product, but such histochemical evidence is notoriously difficult to interpret (Spicer, 1961). With advancing age, large epithelial cysts increase in number and degenerate to form Hassal's corpuscles (Arnesen, 1958), presumably bringing secretory activity to a close.

According to this interpretation of the available data, thymic secretion begins before birth with the formation of small granules, waxes during the first week after birth by shifting to the secretion of mucoid droplets, and wanes slowly, from two weeks after birth, by storing the secretory product in epithelial cysts. Whatever the deficiencies of this hypothesis it seems to relate the course of secretory activity to the known period when the thymus stimulates lymphopoiesis.

By suggesting that large inclusions of PAS-positive material represent a stored secretory product, I have implied that the rate of release of secretion product is variable and subject to control.

The adrenal cortex may be one controlling factor. Injections of ACTH or hydrocortisone induce an acute thymic involution during which the numbers of PAS-positive inclusions and epithelial cysts increase (Baker, Ingle and Li, 1951; Arnesen and Kierulf, 1961; Ito and Hoshino, 1962*a*). Furthermore, the involution produced by several toxic drugs can be prevented by adrenalectomy (Selye, 1936). Even the physiological involution that occurs at parturition has been attributed to stress acting through the adrenal gland (Ito and Hoshino, 1962*a*). PAS-positive inclusions are reported to be most numerous early in involution, when the mitotic activity of lymphocytes is low, and to decrease in number during the regenerative phase when the mitotic activity is high (Baker, Ingle and Li, 1951; Ito and Hoshino, 1962*a*). This apparent inverse relationship between the prevalence of inclusions and the mitotic activity of lymphocytes is made even clearer by Ito and Hoshino's (1962*b*) observations of the changes in the thymus of the mouse during pregnancy and lactation. Late in pregnancy the mitotic activity of thymic lymphocytes was depressed and PAS-positive inclusions were increased. At parturition there was a sudden drop in the number of inclusions, a concomitant increase in Hassal's corpuscles, and a dramatic burst of mitotic activity among the lymphocytes. The authors concluded that Hassal's corpuscles developed from epithelial cysts suddenly emptied of PAS-positive material. These observations all seem consistent with the hypothesis that the PAS-positive inclusions represent stored secretory material that stimulates mitosis of lymphocytes, and that adrenal cortical hormones inhibit its release. Baker, Ingle and Li (1951) concluded that the prolonged thymic involution produced by chronic injections of ACTH could be attributed to an inhibition of mitosis rather than to an increased rate of destruction of thymus lymphocytes.

Csaba, Törö and Bodoky (1963) reported that, 3 and 7 days after an injection of cortisone, there is a depletion of acid muco-

polysaccharide in both thymus and adrenal with a concomitant increase in acid mucopolysaccharide levels in the serum. Thymectomy reduced this increase in serum levels. The apparent release of acid mucopolysaccharide from the thymus into the blood occurred during what Ito and Hoshino (1962*a*) termed the regenerative phase of acute thymic involution, when the mitotic activity of lymphocytes is high and the number of PAS-positive inclusions is decreasing. If these observations can be correlated in a single experiment, it may furnish a valuable tool for measuring the secretory activity of the thymus and the serum levels of its putative hormone.

As a final exercise in speculation, let us re-examine the early changes in thymic cytology in relation to the activity of the adrenal cortex. PAS-positive inclusions first become prominent about two weeks after birth, the time when the adrenal cortex begins to function. Perhaps the onset of adrenal secretion induces a mild, continuous thymic involution that is reflected in the increasing number of PAS-positive inclusions. In other words, the adrenal cortex may moderate thymic secretory activity by inhibiting the release of secretory product. Another event attributable to the initiation of adrenal cortical secretion is that the intestinal mucosa stops absorbing massive quantities of whole protein (Clark, 1959). Shortly after this, the mouse begins to manufacture immune globulins at a rapid rate (Fahey and Barth, 1965). Perhaps all three of these events are related to some role played by the adrenal cortex in the maturation of immunological competence. Suppose, for instance, that by stopping the massive ingress of antigens into the body the adrenal makes it safe for the animal to make antibodies without becoming hypersensitive to dietary proteins. Suppose that, at the same time, by moderating the thymic stimulus to mitosis of the lymphocytes, the adrenal cortex frees them to proliferate in response to antigens.

All through this discussion it has been assumed that the role of the thymic hormone is to stimulate proliferation of lympho-

cytes, but the exact mechanism of its action remains obscure. It was also implied in the preceding paragraph that the thymic hormone, by stimulating lymphocytic mitosis, interfers with the proliferative response to antigens. That this may be true within the relatively secluded environment of the thymus has already been suggested (Clark, 1964), but many other possibilities need to be considered in trying to understand the role of the thymus in the complex responses of the lymphoid system to antigens. The natural history of thymic secretion proposed here on the basis of fragmentary data is hypothetical at best, but it is presented in the hope that it may provide guides to further attempts to study the endocrine function of the thymus.

SUMMARY

In view of the growing evidence that the thymus produces a hormone capable of stimulating lymphopoiesis, the thymuses of young adult mice were examined for cytological indications of secretory activity. In electron micrographs thymus medullary epithelial cells possessed the nuclear and cytoplasmic fine structure of cells that secrete a proteinaceous or mucoid material. In addition, there were three types of cytoplasmic inclusions that might represent stored secretory material: small dense granules, clusters of vacuoles containing an amorphous material and large dense inclusions, both intracellular and intercellular. These structures were basophilic, PAS-positive or both. Radioactive sulphate and [^{14}C]glucosamine, detected by radioautography, were rapidly and preferentially incorporated into the cytoplasm of medullary epithelial cells and into vacuoles and large inclusions. These observations were interpreted to indicate that medullary epithelial cells manufacture and secrete a sulphated acid mucopolysaccharide. Cytological and histochemical examination of the thymus in foetal and suckling mice indicated that secretory activity may develop late in foetal life, accelerate after birth and

moderate about two weeks after birth. On the basis of these observations, it was suggested that the adrenal cortex may play a regulating role in the control of thymic secretion and the maturation of immunological competence.

ACKNOWLEDGEMENT

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DISCUSSION

Metcalf: Dr. Clark, have you investigated the changes in the appearance of the secretory granules in either neonatal or embryonic thymus grafts in adult animals? According to your thesis, there should not be the same time sequence of changes here as you observed in the neonatal animal because the adult host animal would have an intact functional adrenal cortex.

Clark: I agree. I have not done this but I think it is a thing that ought to be done.

Ito: We have made some electron microscopic observations on the epithelial-reticular cells in the thymic medulla. In the hamster thymus, there are characteristic, periodic acid-Schiff (PAS)-positive, colloid-like inclusions in the medullary epithelial cells (Ito, T. [1959]. *Z. Zellforsch. mikrosk. Anat.*, **49**, 739). Such inclusions are also observed in the mouse but they are more prominent in the hamster. We therefore used the hamster to try to determine the nature of these inclusions and to find out what structural elements would be associated with functional activity of the epithelial-reticular cells (Ito, T., and Hoshino, T. [1966]. *Z. Zellforsch. mikrosk. Anat.*, **69**, 311). In the hamster the general structural pattern of the thymus is the same as in the mouse (Hoshino, T. [1963]. *Z. Zellforsch. mikrosk. Anat.*, **59**, 513). In the medulla of the

hamster's thymus two types of epithelial cells can be distinguished by cytoplasmic features, although intermediate forms which are difficult to classify are frequently found. One cell is relatively elongated; the cytoplasm contains abundant ribosomes and ergastoplasm and appears relatively dark, and desmosomes and fibrils characteristic of epithelial cells are sometimes encountered. The other cell is rounder in shape and

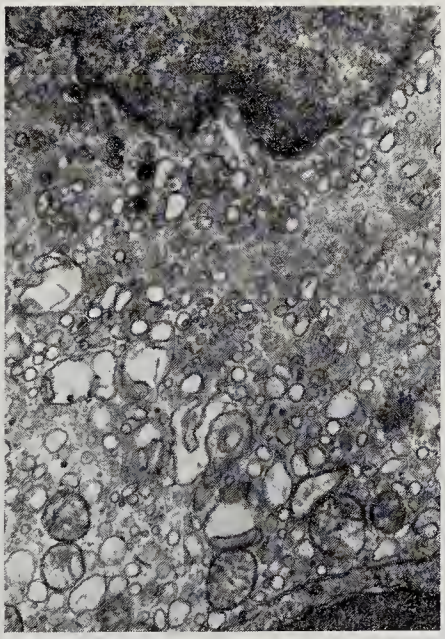

FIG. 1 (Ito). Part of light, epithelial-reticular cell in medulla of thymus of the hamster. The cytoplasm possesses abundant vesicular elements of varying sizes. (× 16,000).

lighter in appearance and a characteristic feature is a striking abundance of vesicular elements. The vesicles, bounded by a distinct membrane, usually accumulate in the cytoplasm (Fig. 1). Larger vesicles are very often intermingled with the vesicular elements so that in certain areas there are numerous vesicles of extremely variable size. In occasional cells single large vesicles, which seem to develop from a coalescence of smaller vesicles and with villous protrusions of the luminal

surface, are seen; the luminal borders of the large vesicles are often ciliated. It is evident that the enormous vesicles seen under the electron microscope correspond to the medullary inclusions revealed by light microscopy. The only cytological elements in the cells that undergo dynamic changes appear to be these vesicular structures, and they seem to be associated with functional activity of the cells. The enormous

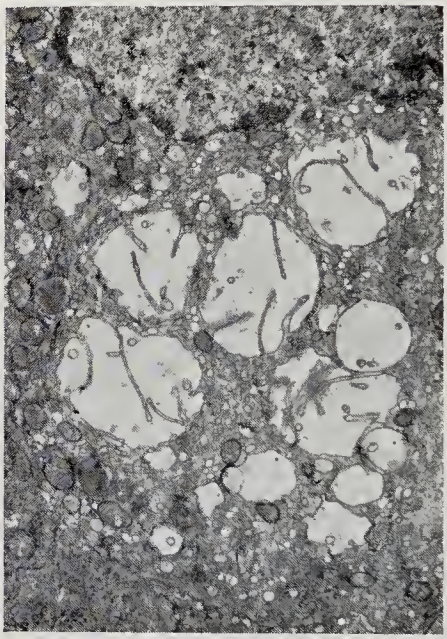

FIG. 2 (Ito). Part of light, epithelial-reticular cell in medulla of thymus of the hamster. Note large vacuoles among the abundant vesicular structures. (× 8800).

vesicles are considered to be a specialized form of vesicular structure. The findings in the thymus of both hamster and mouse are similar (Figs. 2, 3).

Previous electron microscopic studies have been made on the thymus of young adult mice (Hoshino, 1963, *loc. cit.*) and we wanted to find out if the thymus medulla of the newborn mouse shows any morphological evidence for functional activity. The epithelial cells of the medulla of newborn mice possess numerous vesicles of varying sizes

but the enormous ciliated vesicles seen in adult mice are rarely found. In a 17-day-old mouse embryo the medullary epithelial cells contain almost no large vesicles at all.

Clark: Dr. Ito's observation of vesicles soon after birth is certainly consistent with what I have seen.

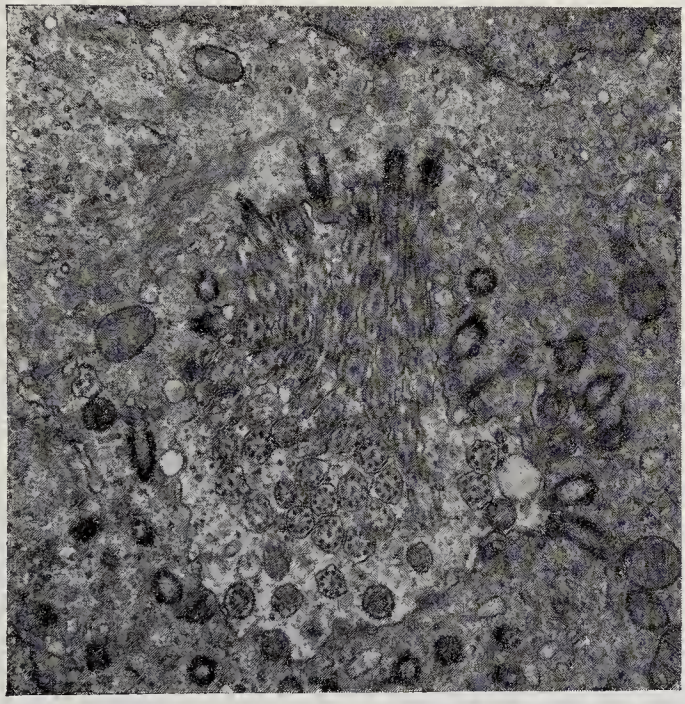

FIG. 3 (Ito). Enormous cileated intracellular cyst in an epithelialreticular cell in thymus of the hamster. (× 17,400).

Gowans: Dr. Clark, could you say something about the relation of the presumptive secretory cells to the blood supply of the thymus? As there appears to be no close relationship between these cells and the blood-vessels would you deduce, on structural grounds, that the postulated thymic hormone may act mainly within the thymus rather than at remote sites after its release into the circulation?

Clark: Certainly these cells do not lie in the characteristic relationship of an endocrine gland to blood vessels, that is closely arrayed along them, but at the same time none of the cells are very far away. If the hormone

is active in lymphopoiesis then it must be active intrinsically. Morphological studies cannot give us direct evidence on whether the hormone acts inside or outside the thymus; grafting studies will be needed to elucidate this problem.

Metcalf: There are, of course, epithelial cells in the cortex of the thymus, as Dr. Clark has pointed out, and these, characteristically, are arranged along the blood vessels. One of the things that has intrigued us is that there is an obvious concentration of mitotic activity in the lymphocytes adjacent to the cortical epithelial cells, and yet in the medulla where there are most epithelial cells there is least mitotic activity (Metcalf, D. [1964]. *In* The Thymus: Wistar Institute Symposium Monograph No. 2, p. 53, ed. Defendi, V., and Metcalf, D. Philadelphia: Wistar Institute Press). Dr. Clark, have your electron microscopic observations shown any difference in fine structure between the two types of cell with respect to secretory products?

Clark: Yes, indeed. The cortical epithelial cells are stretched out rather tenuously, whereas medullary epithelial cells are bulky. Some cortical cells do contain dense inclusions, but these differ from the inclusions in the medullary cells in containing very dense amorphous material. The cortical cells themselves do not concentrate sulphate or glucosamine.

Feldman: The thymus of the adult organism has been shown to maintain its inductive function following total-body X-irradiation: the regeneration of the lymphoid system and of immunological competence has been found to be thymus dependent. In view of this Dr. Clark, have you examined the adult thymus electron microscopically, after exposure to X-irradiation, and does it undergo the same sort of changes as the neonatal thymus? That is, does it pass from a phase of granules, to vacuoles and then to dense inclusions? If such changes in the neonatal thymus are associated with its inductive function one would expect to find similar changes in thymuses of adult animals after irradiation.

Clark: I have not looked for this particular type of change.

Woodruff: Dr. Clark, have you looked at the way these changes evolve if, instead of waiting for the adrenal cortex to start working, you give the animal cortisone from birth? We (Woodruff, M. F. A. [1957]. *Ann. N.Y. Acad. Sci.*, **64**, 792) treated newborn rats with cortisone from the day of birth for varying periods of time and after stop-

ping the cortisone we tried to induce tolerance to homografts. With cortisone we found that we could significantly extend the period in which tolerance could be induced, and we wondered then if this extension could be correlated with any anatomical changes in the animal? We did not study the thymus at that time because little was known about it but, after hearing what you said today, I think it would be interesting to know whether the lengthening of this period during which tolerance can be induced by giving a set dose of antigen would also correlate significantly with the prolongation which occurs when cortisosteroids are given immediately after birth. Have you tried this?

Clark: No. I have studied the absorption of whole proteins in newborn mice (Clark, S. L., Jr. [1959]. *J. biophys. biochem. Cytol.*, 5, 41), and shown that this can be interrupted prematurely by cortisone. A study of the effect of cortisone on the thymus of suckling mice certainly would be a good test of my hypothesis.

Auerbach: Is it certain that the adrenal cortex does not function until two weeks after birth?

Clark: Yes. Florence Moog has examined this question in detail and established that in the rat and mouse the adrenal cortex is small and relatively inactive during this period (Moog, F. [1959]. *In* Comparative Endocrinology, p. 624, ed. Gorbman, A. New York: Wiley and Sons).

Šterzl: Some of our experimental data are relevant to Dr. Clark's hypothesis on the development of immune mechanisms. Newborn and one-month-old piglets (both germ-free animals and those fed with a non-antigenic diet) were immunized with sheep erythrocytes, 5×10^9 cells/kg. of body weight. The number of antibody-forming cells /10^8 splenic lymphoid cells was estimated by the plaque technique. In animals immunized immediately after birth, and at the beginning of the second month of life, similar numbers of antibody-forming cells were detected on the third to the fifth day after immunization; there were about two hundred antibody-producing cells per 10^8 lymphoid cells (Šterzl, J., *et al.* [1965]. *In* Molecular and Cellular Basis of Antibody Formation, p. 463, ed. Šterzl, J. Prague: Publishing House of the Czechoslovak Academy of Sciences). The relationship between the number of immunologically competent cells, the X cells,† and the total number of lymphoid cells did not change during the growth of

† See note p. 36

the animals. A substantial increase in the number of cells able to react to antigen results only from specific antigenic stimulus. If the foetus or newborn animal is injected with a low dose of antigen, although the primary antibody response is not observed, proliferation of the cells activated by antigen, the Y cells,† occurs and this conditions the secondary response.

Clark: Do you mean that if you protect the animal from antigen for about six weeks it has fewer lymphoid cells or the same number as just after birth?

Šterzl: In newborn piglets, and older animals reared under sterile conditions, the proportion of antibody-forming cells to a given number (10^8) of lymphoid cells from the spleen remains constant: if the total number of lymphoid cells increases the number of immunologically competent cells also rises.

We have not found that adrenalectomy affects the onset of antibody-forming capacity. In adrenalectomized newborn rabbits, for example, the number of antibody-producing cells, estimated at three and five days after injection of antigen, was about the same as in control rabbits.

Clark: And yet, during the first week after birth, there is quite a growth of the lymphoid system and an increase in the number of lymphocytes.

Šterzl: Yes.

Good: But not without antigenic stimulation.

Burnet: Dr. Šterzl, are you talking about the number of lymphocytes in the spleen or in the body generally?

Šterzl: In the spleen. In the studies where the relationship between the number of lymphoid cells and the number of antibody-producing cells was estimated, the spleen was used as the source of lymphoid cells. Our calculations of the total number of lymphoid cells in the organism are based on the number of lymphoid cells in 0·1 g. of wet lymphatic tissue from the piglet; this number is known to be $(5–10) \times 10^6$ cells. On the assumption that lymphatic tissue represents about 1 per cent of

† X cells = unprimed, immunologically competent cells; Y cells = primed cells. Z cells = stimulated Y cells. A first dose of antigen converts the X cell into a Y cell; a second dose causes the Y cell to differentiate into a Z cell and to produce antibody (Šterzl, J. [1965]. *In* Molecular and Cellular Basis of Antibody Formation, p. 562, *loc. cit.*).

the total body weight (Yoffey, J. M. [1960]. Quantitative Cellular Hematology. Springfield, Ill.: Thomas), a piglet weighing 1 kg. will have approximately 10^9 lymphoid cells.

Clark: The ratio is not increasing, then, although the total number is. We are simply talking about the proportion that will respond to a given antigen and this is not necessarily relevant to the question of the general proliferation of lymphocytes.

Good: This is very important. Unless there is clear evidence of an alteration in endocrine function (other than thymic) which results from antigenic stimulation, there must be another explanation for the proliferation of lymphoid cells in the periphery when they are not under thymic influence. Another way of putting this is to say that if you withhold antigenic stimulation you withhold growth of the peripheral lymphoid tissue.

Clark: Yes. What I have suggested is that early lymphoid growth may be dependent on the thymus, but that at about two weeks this growth may become almost totally, or at least increasingly, dependent upon antigenic stimulation.

Good: Do you think the cessation of thymic influence, if it occurs, is maturational and completely independent of exposure to antigen, or is the relationship between antibody-producing and total lymphoid cells conditional on this exposure?

Clark: I do not know and I would hate to guess at the moment.

Good: Dr. Clark, are the granules that are formed in the late embryonic period PAS-positive?

Clark: No, they are strongly basophilic.

Good: Do you think that the PAS-positivity—the vacuole formation and the late heavy granule (the dense granule) formation are simply degenerative changes, a sort of degenerative consequence of dealing with some other product of these cells?

Clark: This is certainly a possibility but the small granules found at an early stage in cells that do not contain vacuoles seem to be in close relationship with the Golgi apparatus and have the appearance of being manufactured there.

Nossal: We have all been suspicious in the last few years that the whole of thymic function cannot be explained on the basis of just one hormone. Dr. Clark, have you found morphological evidence for

more than one type of secretory product? Your main thesis has obviously concerned itself with the probable principal thymic hormone, conceivably the lymphocytosis stimulating factor or competence-inducing factor (Miller, J. F. A. P. [1965]. *Br. med. Bull.*, **21**, 111). Have you found any ancillary evidence that might be consistent with a second factor produced by the same general category of cells?

Clark: It seems to me that while it is possible to link the granules and the vacuoles as parts of a continuous chain it is also important to consider that they may represent completely different things. There is a change from predominantly basophilic contents in these structures to more and more PAS-positive staining from about two weeks after birth onward; this might represent a change in hormonal activity. Furthermore, there are the cortical epithelial cells which contain a different sort of inclusion that does not take up sulphate.

Burnet: Dr. Clark, do you consider your results with the sulphate labelling indicate that the hormone concerned is a sulphated polysaccharide?

Clark: I think that my results at least suggest that the cells are making and segregating a sulphated mucopolysaccharide; if this is the hormone then we should certainly be looking for a sulphated mucopolysaccharide.

Burnet: I think this is an important clue; we should look for hormonal activity in sulphated polysaccharides.

Clark: In my presentation I drew attention to the report by Csaba and co-workers (Csaba, G., Törö, I., and Bodoky, M. [1963]. *Z. mikrosk.-anat. Forsch.*, **69**, 467). These workers found that after corticosteroids had produced involution of the thymus, mucopolysaccharides in the serum increased and the acid polysaccharide content of the thymus decreased.

Ford: Are there any other examples of mucopolysaccharides that have been identified with hormonal activity or would the postulated thymic hormone be unique in this respect?

Clark: We must, of course, include the glycoproteins, which brings in the pituitary and many of its hormones.

EMBRYOGENESIS OF IMMUNE SYSTEMS

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In recent years there has been an increasing emphasis on delineating the developmental systems responsible for the rise of immunological competence. Sparked by the concept of self-recognition (Burnet, 1961) and the experimental demonstration of immunological tolerance (Billingham, Brent and Medawar, 1953), numerous workers have attempted to define the conditions whereby the cells of the immune system become regulated or selected during the period of maturation.

In the last few years attention has become focussed on the thymus as one of the controlling elements in the development of immunity (Good and Gabrielsen, 1964; Defendi and Metcalf, 1964; Miller, 1964*a*). Arguing from a series of embryological experiments, the concept of a thymic origin of immunocompetent cells, already well stated by Beard (1900) and experimentally approached by Gregoire (1935), was re-emphasized by Ruth (1960) and Auerbach (1961*a*). In these experiments and in subsequent ones (Auerbach, 1961*b*, 1964*a*, *b*) it was demonstrated that thymus differentiation is essentially autonomous during embryogenesis, that thymus lymphoid cells undergo histogenesis during that period when the embryo is most susceptible to the induction of immunological tolerance, and that the differentiation of thymus lymphoid cell populations is uniquely sensitive to immunological cues.

Simultaneously, a surgical approach to the question of thymic function was employed by Miller (1961) who, by removing the

thymus from neonatal mice, was able to demonstrate that in the absence of the thymus transplantation immunity did not develop in normal fashion. From these experiments, from preliminary ones reported by Martinez and co-workers (1962), and from the work of Arnason and his colleagues (1962) arose a whole series of studies delineating the function of the thymus in neonatal animals (Miller, 1964*a*). The general finding that neonatal thymectomy decreased the ability to reject homografts became coupled with a description of general immunological deficit, albeit variable, depending on different antigens, different strains of animals and different timing of assay (Humphrey, Parrott and East, 1964; Parrott and East, 1962).

Even before the thymus became the focal point of immunogenesis in mammals, the bursa of Fabricius was implicated in the development of immunity in birds (Glick, Chang and Jaap, 1956). Subsequent studies in birds, moreover, suggested that in the chicken there is a dissociation between humoral and cellular immune responses. It was thought that the thymus was responsible for the development of the ability to reject skin grafts, while the bursa was essential for the normal development of the ability to produce antibodies to soluble antigens (Aspinall *et al.*, 1963; Warner and Szenberg, 1964*a*). Interestingly, however, the relation of the bursa to the thymus has rarely been considered. Recent experiments point to a role of the thymus in the development of the ability to produce humoral antibodies in chickens (Chaperon, 1965; Cooper, Peterson and Good, 1965) and this fact, coupled with the relatively weak data on thymic involvement in homograft reactions in chickens, the lack of critical information concerning developmental interrelationships between thymus and bursa, and the demonstrated activity of the bursa in some aspects of cellular immune mechanisms (Warner and Szenberg, 1964*b*) suggest that the postulated dissociation between cellular and humoral immunity may be less fundamental than was originally believed.

Recently studies with diffusion chambers have suggested that both the thymus and the bursa of Fabricius act by secreting a humoral factor which promotes lymphoid cell differentiation (Osoba and Miller, 1963; Levey, Trainin and Law, 1963; St. Pierre and Ackerman, 1965) as previously suggested by Metcalf (1959). At the same time it has been demonstrated that lymphoid development of the spleen proceeds by an immigration of cells from extrinsic sources (Auerbach, 1963, 1964*a*, *b*) and that these migratory cells may have immunological competence (Auerbach, 1965). This raises once again the interesting question of the source of origin of immunocompetent cells, for if the thymus (or bursa) does not send these cells and the spleen does not make them, then they must originate from still another organ, such as, for example, the bone marrow. On the other hand, the fact that immunocompetent cells can enter the spleen does not preclude the possibility that immunocompetent cells can also originate there. The fact that the thymus can function via diffusible factors does not preclude the possibility that it also acts by producing migratory cells (Parrott and East, 1964). The possibility of a dual function of the thymus, moreover, is made more likely both by the demonstration that marked thymus cells can on occasion be seen in other organs (Miller, 1962; Taylor, 1963; Nossal, 1964) and also by the fact that humoral function of the thymus appears to be associated with the epithelial rather than the lymphoid cells of the gland (Miller, 1964*b*).

Against this background, there is a great need to pursue studies of embryogenesis of isolated components of the developing lymphoid system from the non-lymphoid origin to the functional state. While, unfortunately, this has not yet been achieved sequentially *in vitro*, virtually all of the individual steps have been accomplished and it will be the purpose of this paper to describe briefly some of the various experiments performed in our laboratory which have been directed to this goal.

EXPERIMENTS

THYMUS DIFFERENTIATION *in vitro*

The 12-day-old mouse embryonic thymus rudiment can be isolated by dissection and grown in filter-well tissue culture (Ball and Auerbach, 1960). Within 7 days such cultures develop from simple epithelio-mesenchymal rudiments into complex lymphoid organs with large, medium and small lymphocytes as well as reticular and stromal material. Experiments involving trypsin separation of epithelium and mesenchyme have demonstrated that thymic differentiation is the result of an inductive tissue interaction between epithelium and mesenchyme (Auerbach, 1960). By the use of Millipore filters and of marking methods, moreover, it was demonstrated that the mesenchyme gives rise to the stromal elements while the epithelium is the main, if not the only, source of lymphoid cells during embryogenesis of the thymus (Auerbach, 1961*b*, 1964*a*, *b*).

SPLEEN DIFFERENTIATION *in vitro*

When spleen rudiments obtained from 13- to 14-day-old embryos are cultured in similar fashion no equivalent lymphoid development is observed (Auerbach, 1963) (Fig. 1). This result stands in sharp contrast to the results when similar rudiments were grafted into the anterior chamber of the eye of adult mice. In the graft situation complex development occurred within 7 days. It was demonstrated, however, that lymphoid development in such grafts was the result of cell immigration (Auerbach, 1964*b*, 1965) so that the grafting experiments in fact do not contradict the observations made *in vitro*.

BONE MARROW DIFFERENTIATION *in vitro*

When intact femoral rudiments from 15- to 17-day-old embryos are explanted *in vitro* rapid overgrowth of bone occurs and no

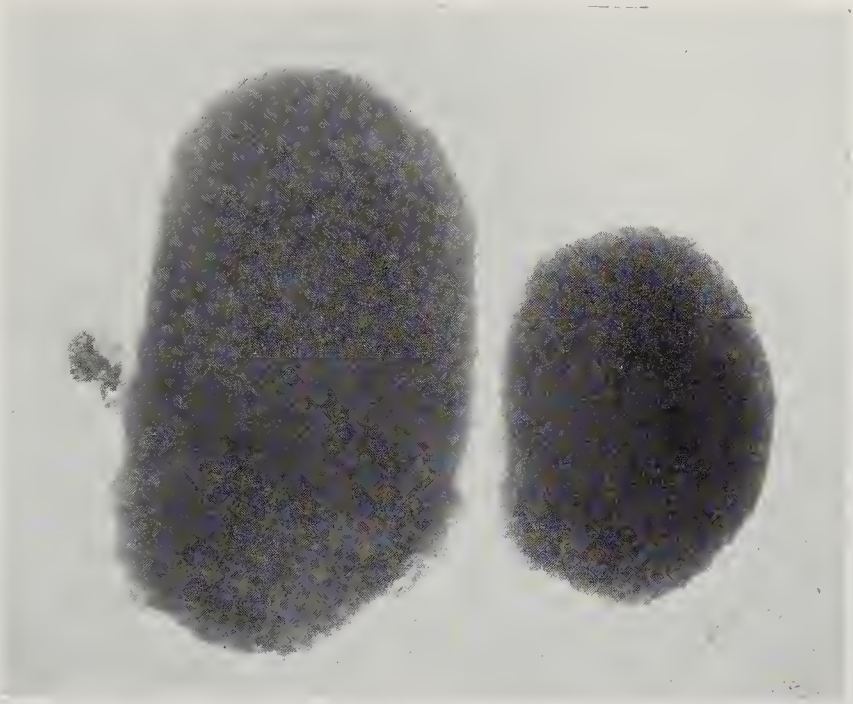

FIG. 1. Graft-versus-host reaction *in vitro*. Note splenomegaly in spleen grown in the presence of parental cells compared with syngeneic control. (× 48).

development of marrow takes place. Isolated central fragments of the femur, on the other hand, can be maintained *in vitro*, and some degree of marrow development takes place under these conditions. Lymphoid cells do not appear, however, and cultures become moribund after 10 to 14 days.

SPLEEN-THYMUS INTERACTION *in vitro*

The thymus becomes lymphoid when isolated but lymphoid development and growth is specifically enhanced by the presence of spleen tissue. This effect can be mediated across an intervening Millipore filter barrier (Auerbach, 1964*a*). A corresponding effect of the thymus on embryonic spleen was not observed.

Recently, spleen explants from neonatal animals were combined with thymus cells. The thymus cells had the effect of stimulating granulopoiesis in the spleen (20 out of 24 cases). The effect was variable in extent and occurred in both syngeneic and allogeneic combinations; this must be taken into consideration in interpreting the results obtained by Hilgard and co-workers (1965) who describe thymus cells as eliciting graft-versus-host reactions.

MARROW-THYMUS INTERACTION *in vitro*

When adult bone marrow is added to embryonic thymus cultures lymphoid cells appear in marrow channels (Globerson and Auerbach, 1964; Auerbach, 1965). Originally restricted to the region of contact between thymus and marrow the cells can ultimately be found throughout the explanted marrow. At the same time, the thymus appears to have a stimulatory effect on granulopoiesis.

MARROW-SPLEEN INTERACTION *in vitro*

The addition of adult bone marrow to embryonic spleen rudiments leads to the subsequent localization of lymphoid cells within the spleen (Fig. 2) (Globerson and Auerbach, 1964; Auerbach, 1965). This is particularly intriguing since embryonic spleen by itself does not become lymphoid under our conditions of explantation, and marrow isolated alone fails to maintain lymphoid cells.

When bone marrow from 17-day-old embryos is added to embryonic spleen rudiments no effect has been noticed. Similarly, if the central fragment of the femur, including the inner presumptive marrow, is surrounded by spleen tissue and then cultured, no lymphopoiesis takes place. Marrow-spleen interaction can thus only be demonstrated after the marrow contains lymphoid cells of its own.

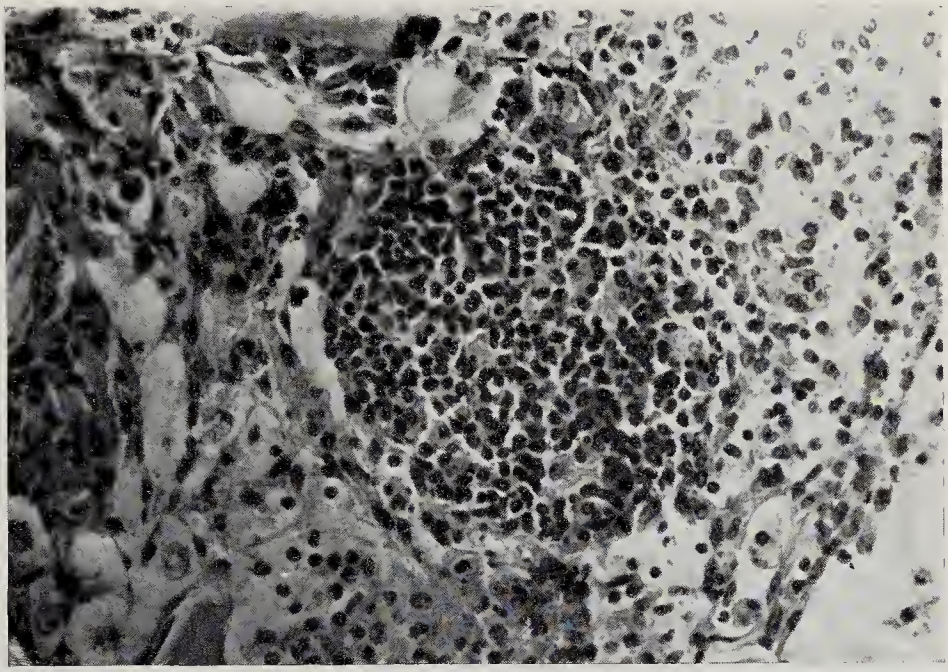

FIG. 2. Combination culture of embryonic spleen and adult bone marrow. Note lymphoid centre. (× 515).

MARROW-SPLEEN-THYMUS INTERACTION *in vitro*

Finally, three-way combinations have been made including embryonic spleen and thymus and either embryonic or adult bone marrow. When embryonic spleen and thymus are combined with adult bone marrow, complex organized lymphoid cultures result (Auerbach, 1965). The marrow is extremely active, being both lymphoid and filled with granulocytes, the thymus is high in mitotic activity, and the spleen becomes quite lymphoid in appearance (Figs. 3 and 4).

When pre-lymphoid marrow (central femur explants) is combined with pre-lymphoid spleen and thymus, moreover, a similar, although not as striking, effect is seen. In this combination also both the marrow and the spleen become lymphoid.

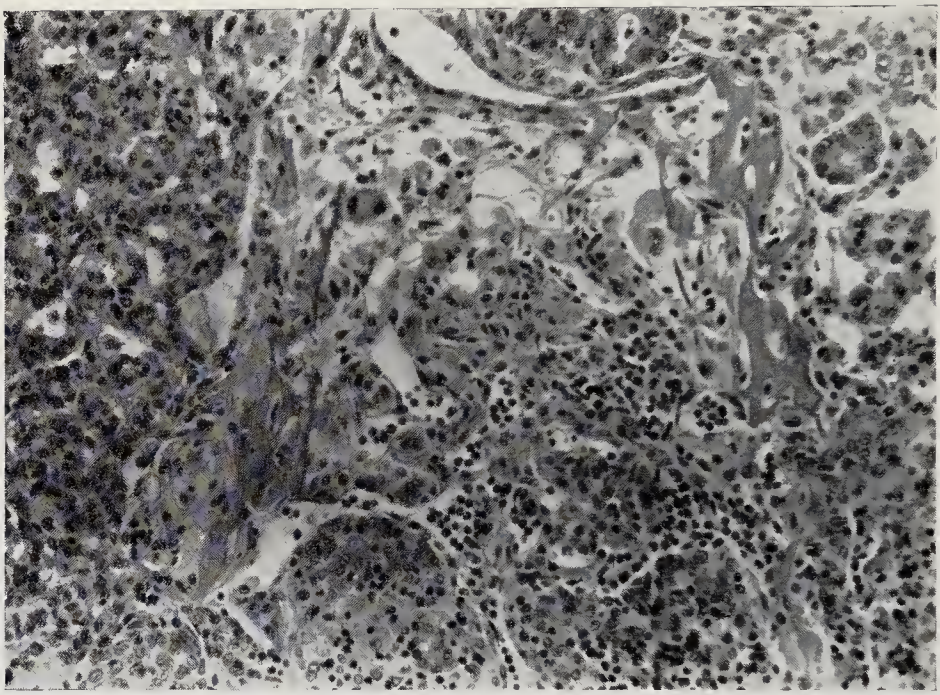

FIG. 3. Combination culture of embryonic spleen and thymus, with adult bone marrow. Histological preparation, (× 820).

FIG. 4. Combination culture of embryonic spleen and thymus, with central part of embryonic femur. Living culture, (× 48).

FUNCTIONAL TESTS OF COMBINATION CULTURES

It has been demonstrated that graft-versus-host reactions can be produced in *in vitro* systems (Auerbach and Globerson, 1966; Globerson and Auerbach, 1965). To use this procedure for assay of immunological function in combination cultures, spleen, thymus, and femur fragments from 17-day-old C3H or $(C \times C3H)F_1$ embryos were grown for 6 days after which time the cells were harvested and used as test cells. When parental C3H cultures were tested, they were able to produce splenomegaly in both $(C \times C3H)F_1$ hybrids and in neonatal allogeneic C57BL spleen explants, while the $(C \times C3H)F_1$ test cells were able to produce splenomegaly in allogeneic C57BL spleen explants, but not in syngeneic $(C \times C3H)F_1$ fragments. Since other experiments had previously demonstrated that parental-strain embryonic spleen, thymus, and marrow individually did not have the competence to elicit the splenomegaly reaction, it may be concluded that maturation of the immune system, as judged by the ability to give a graft-versus-host reaction, has taken place *in vitro*.

The fact that the combined rudiments can mature *in vitro* strengthens the suggestion made previously that the thymus, bone marrow and spleen may all participate in some interdependent way in the development of the immunologically-competent animal. A number of simple experiments can now be performed with the *in vitro* systems described in this paper, which should more clearly define the precise role played by each of these organs in the maturation process.

SUMMARY

Experiments are described involving tissue-culture study of mouse embryonic thymus, spleen, and bone marrow rudiments. An attempt is made to correlate histogenesis with functional maturation. It is suggested that interactions of bone marrow, thymus and spleen are all essential for immunological maturation.

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DISCUSSION

Feldman: In vivo experiments carried out in our own and other laboratories have demonstrated that the thymus can induce differentiation of cells of extrinsic origin to lymphoid cells. Cells of bone-marrow origin injected into X-irradiated animals may differentiate to lymphoid cells following the inductive effect of the thymus, and such cells have been shown to be immunologically competent. I would like to ask Dr. Auerbach if the thymic lymphocytes which he showed us had developed from the thymic epithelium had achieved immunological competence?

Auerbach: We have tried to find this out, but we have been unsuccessful in our attempts. We are now doing extensive further studies, as controls for the combination studies, but so far we have not been able to show immunological competence of the thymic epithelial-lymphoid cells. Dr. A. Globerson and I (1964. *Science*, **146**, 425), working

with irradiated spleen and bone marrow, have found that adult bone marrow will restore irradiated spleen *in vitro*. The thymus enhances this restoration.

Gowans: What exactly does the thymus restore in this system?

Auerbach: It restores lymphoid cellularity, and it may restore functional activity which can subsequently be tested by the graft-versus-host reaction *in vitro*.

Kaplan: In 1958, Irwin Berman and I (unpublished material) observed what appeared to be a humoral effect of bone marrow on the capacity of the thymus that had been infused by radiation to regenerate. This is a difficult system to study *in vivo*. There have been suggestions that this effect is due to actual migration of cells from the bone marrow into the thymus. We did have some early success—unfortunately difficult to reproduce regularly—by putting bone marrow into diffusion chambers and assaying for the regeneration of the thymus in irradiated test animals. I think, Dr. Auerbach, that your system might lend itself very well to this experiment and I wonder if you have done any studies along these lines.

Auerbach: Dr. Globerson and I (and she has done more of this work than I have) have studied the effect of urethane on the thymus. When we culture embryonic or newborn thymus, the addition of urethane completely inhibits further differentiation. The thymus loses its lymphoid cells and remains inactive. The urethane is removed after one or two days but there is never any restoration of lymphoid cells. But when we add bone marrow, within four days the thymus looks completely normal. When the bone marrow is added through a Millipore filter the restoration is at least as good, or better, than when it is added to the thymus preparation directly. This indicates that whatever the bone marrow is doing *in vitro* it can operate across a Millipore filter barrier (Globerson, A., and Auerbach, R. [1965]. *In* Wistar Institute Symposium Monograph No. 4, p. 3. Philadelphia: Wistar Institute Press). We can do the same thing with the lung, that is, we can produce urethane-induced lung defects. Bone marrow has no restoring effect on the lung but embryonic mesenchyme does. It appears that restoration from the effects of urethane is quite specific: the bone marrow can restore thymic deficiencies but not certain other urethane-induced abnormalities, whereas mesenchymal cells may be effective

for some tissues, such as lung, but cannot restore the urethane-damaged thymus.

Klein: Dr. Auerbach, are the parental or the hybrid cells proliferating in your *in vitro* splenomegaly system? Are any of the cells killed in the course of the reaction? Is close contact necessary when the cells are separated by a filter?

Auerbach: The reaction can occur through a filter but is not as pronounced as when the cells are in direct contact. By about four days the target is no longer able to produce splenomegaly because by this time it is mature and a splenomegaly reaction can only occur when the host animal cannot react against the donor. Let me try to clarify this. First of all, it is a little confusing to talk about a graft-versus-host reaction *in vitro*. I will explain what I mean by this. We put a piece of spleen into a culture dish and call this our "host"; normally this host is newborn, F_1 spleen. We add to this adult, F_1 cells (the "donor") and splenomegaly occurs when the parental cells are reacting against the F_1 host. If we now use adult cells from a C strain of mouse and spleen fragments from a newborn C3H mouse as graft and host respectively, the adult C cells react against the C3H cells and, again, cause splenomegaly. However, the host C3H spleen fragment does not react against the C cells because it is immature. If we do this experiment using a four-day-old C3H host, then splenomegaly does not occur.

Klein: Why not?

Auerbach: Presumably because the spleen in this situation is competent and will destroy the donor cells, or prevent their functioning before they can stimulate splenomegaly.

Klein: What happens if you use F_1 adult and parental strain embryonic cells?

Auerbach: In this situation there is no splenomegaly. We are well aware of the work done in Stockholm in this area (Möller, E. [1965]. *Science*, **147**, 873). We have repeated this work using parental host and F_1 donor cells and in a system of this sort we have not produced splenomegaly. The results might well be different if we kept the preparation for eight or ten days and transferred, or stimulated and then retransferred, the cultures.

Woodruff: Could you please repeat the details of all this?

Auerbach: When we used F_1 adult cells and parental-strain embryonic or newborn tissue, splenomegaly did not occur. I do appreciate the possibility that splenomegaly in the non-specific sense could arise, as reported from Professor Klein's laboratories.

Klein: Our reaction *is* specific, but we are using the word in a different sense from you.

Auerbach: All right, I agree!

Klein: Could the foreign specificity give the immune reaction throughout these experiments?

Auerbach: I do not think that this is so for the splenomegaly in our system. The splenomegaly reaction itself is extremely complicated as you know.

I can, however, answer your question about whether host or donor cells are proliferating. We can use the Grobstein method (Grobstein, C. [1956]. *Expl Cell Res.*, **10**, 424), and employ a Millipore-filter culture system. We put the test spleen on one side of the filter, and glue it in place with a plasma clot. We then add the test host cells on the other side, and in this way we can study host and donor components. In this situation splenomegaly occurs but it is not as extensive as in the direct combination experiments. In the direct combination studies, after two days, the spleen is filled with lymphocytes which then disperse and ultimately disappear. In the Millipore filter system, the spleen is not filled with extra lymphocytes but granulopoiesis goes on and the tissues increase in size. This system can be enhanced by preimmunizing the cells *in vivo*, confirming the results of A. P. Dalmasso, C. Martinez and R. A. Good (1962. *Proc. Soc. exp. Biol. Med.*, **110**, 205) that a weak reaction can be enhanced by preimmunization. The host reaction here is entirely granulopoietic and the donor reaction is lymphoid. These results agree with the results of other workers concerning the host and donor components (Zeiss, I. M., and Fox, M. [1963]. *Nature, Lond.*, **197**, 673; Seto, F., and Albright, J. F. [1965]. *Devl Biol.*, **11**, 1). It seems that splenomegaly is a response involving division, and perhaps this is why the reaction of agglutinins and haemolysins requires phytohaemagglutinin whereas the splenomegaly response does not. This is probably best explained by the results of F. Bach and K. Hirschhorn (1964. *Science*, **143**, 813) with two sets of lymphocytes. In their system test cells are probably actively dividing, but with soluble

antigens division must be induced. In a cellular system such as I have just described we do not need any of these aids—neither phytohaemagglutinin nor presensitization nor direct contact.

Good: Leaving this elegant work in tiny discrete systems to return to the clinical situation, several groups have made pertinent observations here (Hitzig, W. H., Kay, H. E. M., and Cottier, H. [1965]. *Lancet*, **2**, 151; Rosen, F. S., Gitlin, D., and Janeway, C. A. [1962]. *Lancet*, **2**, 380). In the Swiss type of agammaglobulinaemia there is a complete absence of small lymphocytes and a vestigial, completely undeveloped thymus. Attempts have been made to reconstitute babies with this fatal disease by giving thymic transplants but these have had absolutely no effect. In one child Hitzig himself has given multiple thymus transplants without effect, but with foetal liver injections he has been able to reconstitute the lymphocyte population although the baby did not develop immunological competence. Rosen and Janeway (1965. Personal communication) have made comparable observations after giving adult bone marrow to these babies. What is needed here may be what Dr. Auerbach has just described, that is, interacting systems to mature the foetal lymphoid haemoblasts. The thymus perhaps should be transplanted and even the spleen as well.

Auerbach: This is a very interesting suggestion. We are just starting to work with foetal liver to find out what effects it has in combination with thymus or spleen or bone marrow.

Šterzl: Dr. A. Friedenstein in Moscow (1961. *Acta anat.*, **45**, 31; 1962. *Nature, Lond.*, **194**, 698) demonstrated that the injection of an extract of transitional epithelial cells from the bladder mucosa may induce extramedullary myelopoiesis. Some factor is secreted by these epithelial cells when they are cultivated in a diffusion chamber and this factor induces ectopic bone formation. Dr. Auerbach, could this substance be an inductor or a hormone initiating differentiation of the mesenchyme and thus similar to the substance produced by the thymus?

Auerbach: Drs. M. Feldman and A. Globerson (1964. *Ann. N.Y. Acad. Sci.*, **120**, 182) have discussed in detail the inductive nature of the thymus. I think that Dr. Šterzl's comments are quite logical and Dr. Clark's material also looks relevant and interesting in this context. R. Levi-Montalcini and P. U. Angeletti (1965. *In* Organogenesis, p. 181, ed. DeHaan, R. L., and Ursprung, H. Hew York: Holt, Rinehart

and Winston), in their work on growth factors in the salivary gland, suggest that there are not one but many inductors. There may be a mesenchymal factor, an epithelial factor, a nerve growth factor and various others all of which seem to come from one gland although they may not all come from the same cells. When all these factors have been identified and traced to the various cells there still remain some cells which have no apparent function. It seems that the thymus has a very complex series of actions, and inductive potential may be just one of them.

Dameshek: Dr. Auerbach, will you comment on the relationship of the thymus to bone-marrow elements other than lymphocytes? Do you think that the thymus has anything to do with the production of, say, nucleated red cells, granulocytes and the like?

Auerbach: I refuse to comment on this because I am sure that anything I say will be entirely incorrect in a year's time!

Miller: Myelopoietic elements have been observed in the thymus *in situ* after depletion of lymphocytes by irradiation (Harris, P. F. [1958]. *Br. J. exp. Path.*, **39**, 557) or by cortisone (Craddock, C. G. [1964]. Personal communication). Erythropoiesis has also been described in the thymus of mice in the perinatal period (Albert, S., Wolf, P. L., and Pryjama, I. [1965]. *J. reticuloendothelial Soc.*, **2**, 30). We have also observed myeloid foci in thymus implants in certain experimental situations. When lymphoid differentiation occurs, as in normal syngeneic grafts, no myeloid cells are seen. When lymphoid differentiation does not occur, as in allogeneic grafts, or is delayed, as in irradiated thymus implants, many foci of myeloid cells are seen in the thymus implant (Dukor, P., Miller, J. F. A. P., House, W., and Allman, V. [1965]. *Transplantation.* **3**, 639). In opossums (*Didelphis virginiana*) thymectomized in embryonic life there is a failure of development of the lymphatic tissue but myeloid tissue in the lymph nodes and spleen not only persists but is greatly increased in amount at a time when it would normally have decreased (Miller, J. F. A. P., Block, M., Rowlands, D. T., Jr., and Kind, P. [1965]. *Proc. Soc. exp. Biol. Med.*, **118**, 916). There may thus be an inverse relationship between differentiation along lymphoid pathways and along myeloid pathways within the lymphoid system under certain experimental conditions.

Auerbach: We have found increased granulopoiesis in the marrow in

our preparations when thymus is present but I do not know what this means. We have also observed increased granulopoiesis in the spleen in our spleen-marrow interactions. We have tried to produce graft-versus-host reactions with embryonic thymus injected into newborn animals, and we were very excited to find that embryonic thymus at certain stages can produce splenomegaly. We also found that syngeneic thymus had an effect: this was entirely granulopoietic and immunological competence was not induced. Professor Good also has done some work on this (Good, R. A. [1965]. *Nature, Lond.*, **205**, 143). I would like to hear his views on the granulopoietic but *non-immunological* effect of the thymus on spleen and bone marrow.

Good: We have been working with fully dispersed cells and using controls for the syngeneic thymus. The effect of the newborn thymic cells themselves seems to be an immunological one and not related to the non-specific, thymus-stimulating effect, which is probably what gives rise to splenomegaly in your experiments.

Metcalf: There may be considerable species difference in the influence the thymus has on the proliferation of myeloid and erythroid cells. We were interested in this in the mouse because thymectomized mice, late in life, may develop myeloid leukaemia and reticulum-cell sarcoma. We have found that thymectomy, in the adult mouse at least, has no observable effect on the proliferative patterns of myeloid cells, reticulum cells or monocytes, as studied by tritiated thymidine radioautography; multiple-thymus-grafted animals are also indistinguishable from the normal (Metcalf, D. [1965]. Unpublished observations). Although our findings have been negative this might not be so with thymectomy in mice in the neonatal period, or with thymectomy in other species.

Good: A particularly pertinent clinical syndrome is that in which overgrowth of the epithelial stroma of the thymus is associated with either pure red cell agenesis, or red cell agenesis together with pancytopenia, due to failure of proliferation of cells within the bone marrow. We have been interested in this syndrome in connexion with neonatally thymectomized animals into which a newborn spleen has been transplanted. The newborn spleen is loaded with extramedullary haemopoietic centres, and such a spleen, in a neonatally thymectomized animal, does not develop lymphoid tissue nor do its erythropoietic

and myelopoietic centres involute. We are now introducing thymic extract into these animals to see if this will induce loss of the non-lymphoid haemopoietic tissue. Another possible explanation for this failure of development is that it is dependent upon the influx of lymphoid cells into the spleen. Certainly the syndrome that I have just described, of which there are some fifty recorded instances (reviewed by Schmid, J. R., *et al.* [1965]. *Cancer*, **18**, 216), is really a clear-cut experiment of nature which has not been fully interpreted. This association of abnormalities must reflect some important influence: it cannot be explained by chance alone.

Dameshek: The association of pure red cell and other types of hypoplastic anaemia with thymomas can certainly be described as an experiment of nature, but I think that we should consider an additional interpretation for the mechanism of its production. Recent work suggests that this syndrome may be induced by an immunological mechanism, that is, by the development of an auto-antibody against erythropoietin (Jepson, J. H., and Lowenstein, L. [1966]. *Blood*, **27**, 425).

Good: I agree. I was not suggesting that mine was the definitive interpretation. I do think, however, it is entertaining that removal of the thymic tumour, which is composed almost entirely of stromal epithelial cells, has resulted in remission of the anaemia in about 50 per cent of these cases.

Rowley: Dr. Auerbach, in your experiments on graft-versus-host reactions *in vitro*, you said that you could get a positive reaction with not more, and possibly less, than 10^4 adult spleen cells. This implies that one cell at least in this number is competent to react against the antigens. Have you been able to quantitate your plating techniques, in which you added complement, in the same way and, if so, can you say how many competent cells there are in these preparations?

Auerbach: Do you mean in the control experiments?

Rowley: No, in your experiments with bone marrow and spleen.

Auerbach: No. We have not been able to do this with the spleen fragments. When we test the graft-versus-host reaction we add a cell suspension to our system; when we test for the ability to react against sheep red blood cells we are using organ cultures. In the former experiments, organ cultures are being used to produce antibodies and so we have to keep the cell structure intact; in the latter,

we are using these same organ cultures to demonstrate splenomegaly so the number of cells is not important. In the second set of experiments we know neither how many cells there are nor how many cells within the spleen are actually lymphocytes.

Rowley: Have you tried to break down your splenic material into smaller fragments?

Auerbach: Not yet. We would like to get the reaction in cell suspension first.

THE ORIGIN AND FUNCTION OF LYMPHOCYTES

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A considerable body of evidence combines to show that the presence of the thymus during early life is essential for the normal development of lymphoid tissue and for the maturation of immunological responsiveness. It is likely that the thymus also continues to function into adult life, for thymectomy in adults seriously impairs the return to normal responsiveness of heavily X-irradiated (Cross, Leuchars and Miller, 1964), and of immunologically tolerant, animals (Taylor, 1964). The influence which the thymus exerts on the development of immunological function is correlated at a cellular level with the accumulation in lymphoid tissue of new dividing cells and of small lymphocytes. Experiments employing chromosome markers suggest that many of the dividing cells originate in the bone marrow and reach lymphoid tissue after a period of residence in the thymus (Harris and Ford, 1964; Harris *et al.*, 1964). It is not known whether the thymus also exports small lymphocytes or whether these can arise by division in lymphoid tissue from thymic-derived precursors. The observation that thymic-derived cells in the lymph nodes and spleen of mice are fully competent to initiate graft-versus-host reactions supports the idea that small lymphocytes can originate by one of these two processes (Taylor, 1963).

A large fraction of the small lymphocytes in adult animals are long-lived cells which recirculate continually from blood to

lymph. Experiments with radioactively-labelled small lymphocytes in the rat have shown that the thymus is excluded from this process of recirculation (Gowans and Knight, 1964). Whether any of the new small lymphocytes which slowly replenish the recirculating pool are derived from the thymus has yet to be established, but whatever the contribution of the thymus in the adult animal it is clear that small lymphocyes can also have an independent origin in the lymph nodes and spleen during immunological reactions. The evidence for such a process and its functional implications will be briefly considered.

PRODUCTION OF SMALL LYMPHOCYTES DURING IMMUNOLOGICAL REACTIONS

It has been shown that when an inoculum of small lymphocytes prepared from the thoracic duct lymph of rats is injected into lethally X-irradiated mice, large pyroninophilic cells develop in the white pulp of the spleen by a process which does not involve cell division. A period of rapid proliferation then takes place in which chromosome analyses showed virtually all the dividing cells to be "rat" in origin; and the administration to these mice of a single injection of tritiated thymidine confirmed that the class of cell which begins to divide in the spleen after 24 hours is the large pyroninophilic cell. These findings established that, in a graft-versus-host reaction, small lymphocytes enlarge into pyroninophilic cells which divide (Gowans, 1962; Gowans *et al.*, 1962). In these experiments, the progeny of the large pyroninophilic cells was identified radioautographically in histological sections of spleens as a population of lymphocytes of progressively decreasing size; the precise morphological identification of the cells presented some difficulty. This difficulty was resolved in the following experiments.

Groups of male CBA mice, 3–6 months of age, were injected intravenously with 10^8 rat small lymphocytes approximately

24 hours after 1000 rads of whole body X-irradiation. The "purified" inocula of small lymphocytes had been prepared from thoracic duct cells by the method of Gowans (1962). Intravenous doses of tritiated thymidine, each of 1 μc/g. body weight (specific activity 3 curies/m-mole) were given to the mice at various times after the injection of lymphocytes and radioautographs of the spleens were subsequently prepared with Ilford K5 emulsion. Two types of preparation were examined: histological sections from tissue fixed in formol alcohol and smears of cells made after teasing fragments of spleen in rat serum.

In a preliminary experiment three groups of four mice were killed one hour after a single dose of tritiated thymidine given either 12, 16 or 24 hours after an injection of rat lymphocytes. Radioautographs showed no labelled small lymphocytes in the spleens from any group but many of the large pyroninophilic cells which were present at 24 hours had begun to synthesize DNA. Thus, during the evolution of large pyroninophilic cells from small lymphocytes DNA synthesis starts only after considerable cell enlargement has occurred: small lymphocytes, as such, do not incorporate tritiated thymidine.

Each member of another group of ten irradiated mice was given three doses of tritiated thymidine between 24 and 54 hours after an injection of rat lymphocytes, that is, when the dividing cells in the spleen were virtually all "rat" in origin. The spleens were excised two hours after the last dose of tritiated thymidine and the smears prepared from them showed the presence of many lymphoid cells of varying size with a strongly basophilic cytoplasm which was sometimes vacuolated. Many of these cells were labelled (Fig. 3) and they corresponded to the labelled pyroninophilic cells which were observed in large numbers in histological sections prepared from the same spleens (Figs. 1 and 2). In order to determine the progeny of the labelled cells, a total of 120 million cells was pooled from the ten chimaeric mouse spleens and injected intravenously into a rat which was syngeneic with the

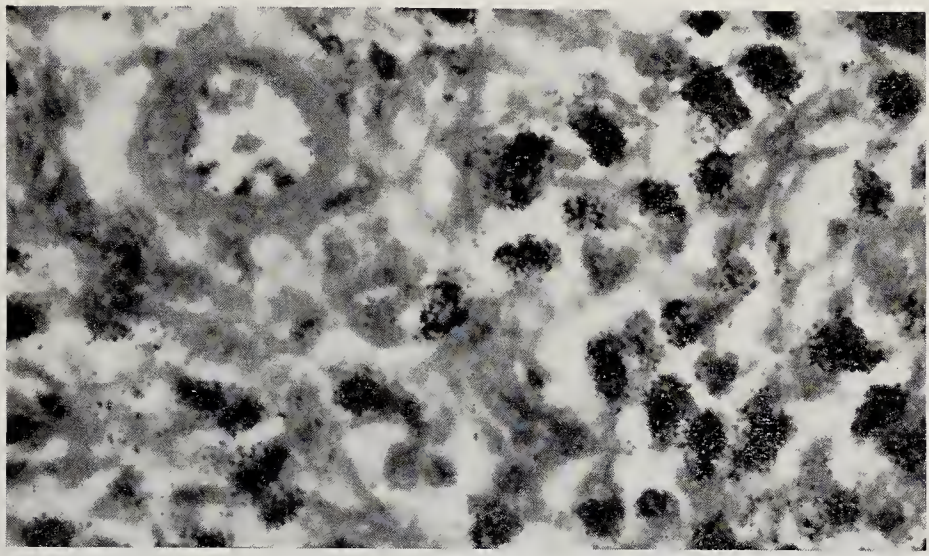


FIG. 1. Radioautograph of spleen from lethally irradiated mouse 56 hours after intravenous injection of rat small lymphocytes and 2 hours after last dose of tritiated thymidine. Most of the large pyroninophilic cells are labelled. Exposure 42 days. Methyl-green pyronin. (× 800).

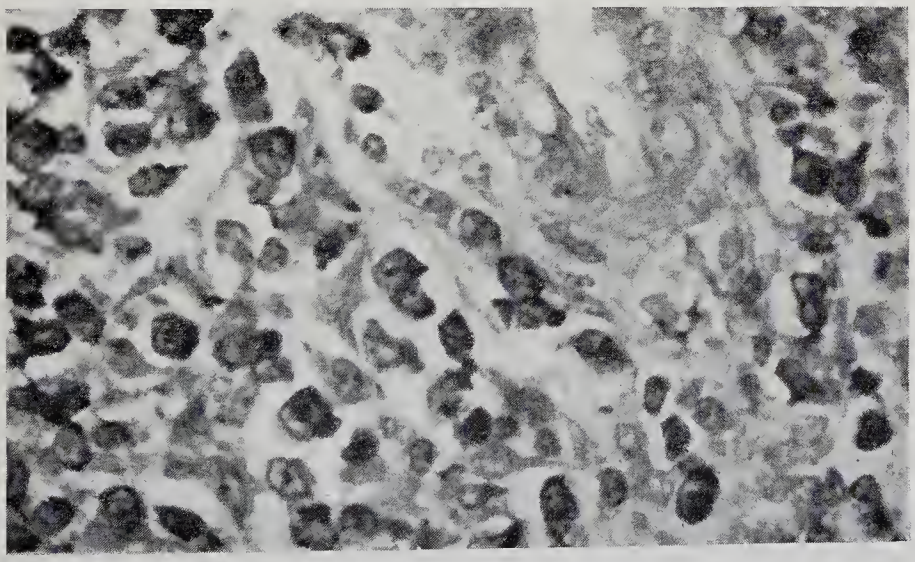


FIG. 2. Histological appearance of spleen shown in Fig. 1. Many large pyroninophilic cells surround the central vessel in the white pulp. Methyl-green pyronin. (× 800).

original lymphocyte donor and whose thoracic duct had been cannulated 24 hours previously. During the next 48 hours substantial numbers of labelled small lymphocytes emerged from

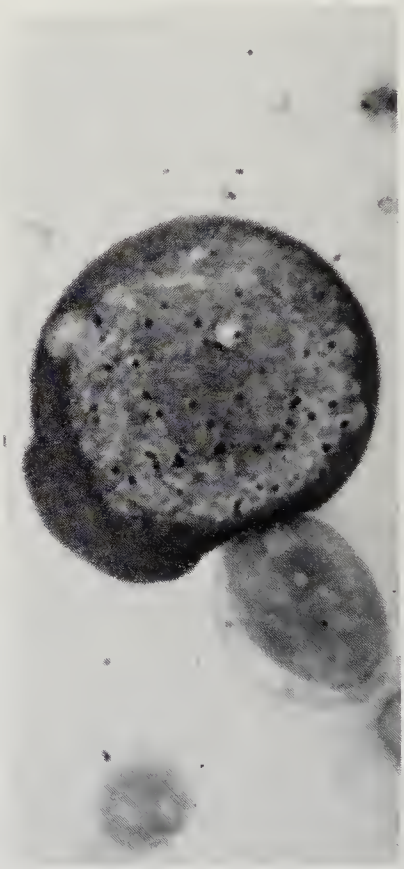
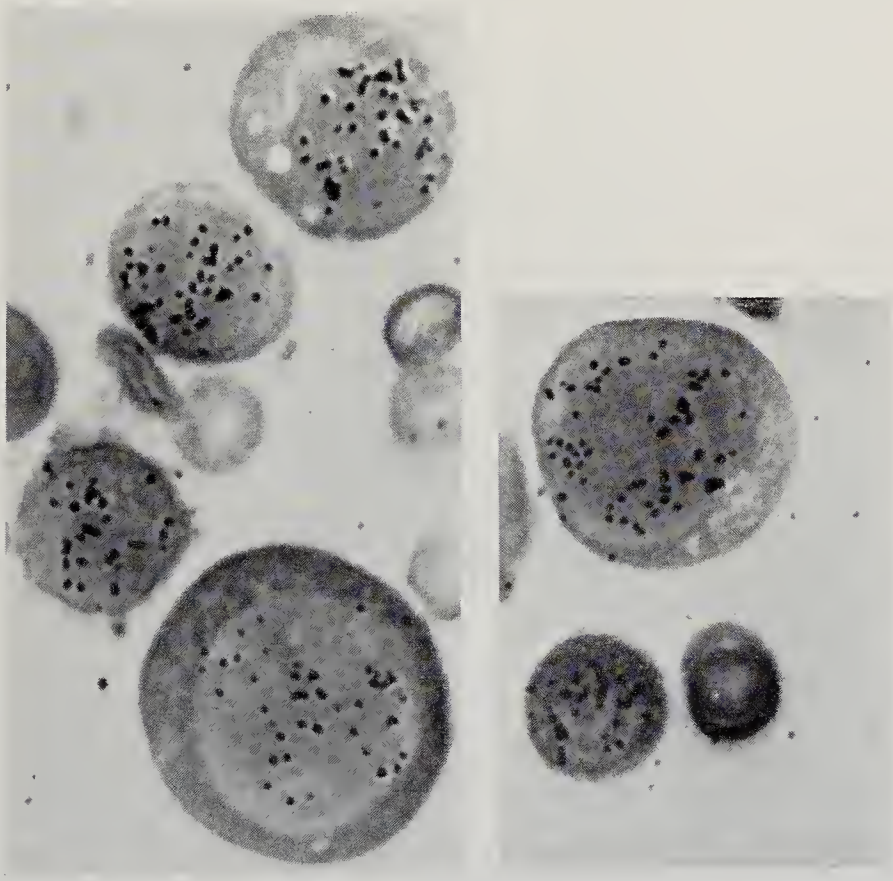


FIG. 3. Labelled large lymphoid cell in smear from spleen shown in Fig. 1. These cells are derived from rat small lymphocytes. Exposure 7 days. Leishman. (× 2000).

the thoracic duct of this rat (Fig. 6). The experiment established that the labelled pyroninophilic cells which were derived originally from small lymphocytes had divided in the rat to produce labelled small lymphocytes. Not only were these labelled cells

identified as small lymphocytes from their morphological appearance, but it could also be claimed that they behaved as lymphocytes since they had found their way into the lymph of the recipient animal.



FIGS. 4 and 5. Labelled lymphoid cells of varying sizes in smear of spleen from lethally irradiated mice killed 80 hours after intravenous injection of rat small lymphocytes and 26 hours after last dose of tritiated thymidine. Exposure 28 days. Leishman. (× 2000).

In a final experiment, the intermediary stages between the large pyroninophilic cell and the small lymphocyte were studied in a further group of ten lethally irradiated mice. These were sacrificed serially after an identical schedule of injections of rat

small lymphocytes and of tritiated thymidine. An examination of smears from the spleens of these mice showed that the labelled cells which were derived from the large pyroninophilic cells composed a family of lymphoid cells of progressively decreasing size (Figs. 4 and 5).

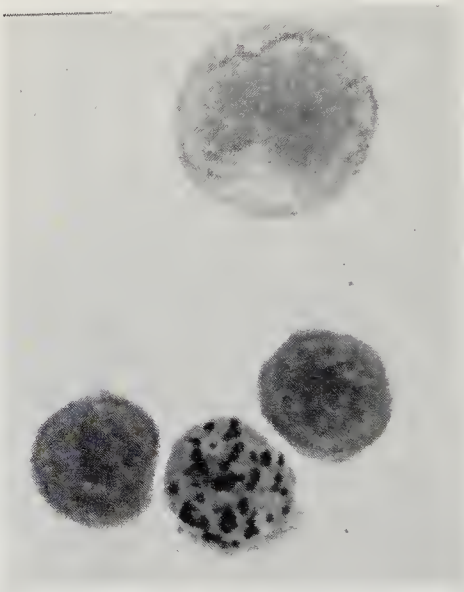


FIG. 6. Labelled small lymphocyte in lymph of rat 48 hours after intravenous injection of suspension of cells from spleens shown in Figs. 1 and 2. Exposure 28 days. Leishman. (× 2000).

It is of some interest to speculate on the possible significance of the production of small lymphocytes during immunological reactions. It has been suggested that the sequence small lymphocyte → large pyroninophilic cell → small lymphocyte might represent the cellular basis of homotransplantation reactions (Gowans, McGregor and Cowen, 1963; Gowans and McGregor, 1965). Small lymphocytes can initiate graft-against-host reactions in rodents and also probably the reaction against orthotopic homografts of skin; in both these situations the production of large

pyroninophilic cells in lymphoid tissue is a hallmark of the reaction. It seems plausible to suggest that the progeny of the large pyroninophilic cells may be the effector cells which have been postulated as the agents which destroy homografts. This scheme leads to the idea that two functional classes of small lymphocytes may exist: those which possess the machinery for effecting a specific response (committed cells) and those which are potentially capable of initiating a response (competent or uncommitted cells). The problem of whether individual competent cells are genetically pre-adapted to react only to certain specific antigens is still unresolved.

New small lymphocytes are also produced in lymphoid tissue during antibody formation. Thus, Nossal and Mäkelä (1962) noted an increased formation of small lymphocytes in the popliteal lymph nodes of rats during a secondary response to *S. adelaide*, and Miller (1964) described the prolonged retention of labelled small lymphocytes in the regional nodes when tritiated thymidine was given to rats after both primary and secondary immunization. The long potential life-span of small lymphocytes both in rodents (Little *et al.*, 1962) and in man (Buckton and Pike, 1964) makes it attractive to suggest that immunological memory may be carried by small lymphocytes generated during immunization.

The experiments of Cohen and Talmage (1965) support the idea that small lymphocytes may be the carriers of immunological memory. These workers showed that the precursors of antibody-forming cells in a secondary response in mice failed to incorporate tritiated thymidine just before secondary stimulation. More evidence on this point has come from the experiments of Uhr and Gowans (1966) on the antibody response of rats to the bacteriophage φX 174. A single subcutaneous administration of 10^{11} plaque-forming units (PFU) gave a low antibody response over the following three weeks, but a brisk secondary response could be elicited when the animals were subsequently

challenged intravenously with 10^{10} PFU (Table I). In order to determine whether small lymphocytes played any part in the secondary response to φX the thoracic ducts of a number of primarily immunized rats were cannulated ten weeks after immunization. The thoracic duct cells were incubated *in vitro* at 37°C for 24 hours in order to eliminate most of the large dividing lymphocytes (Gowans, 1962), and the resulting suspensions of small lymphocytes were injected into syngeneic recipients 24

TABLE I

ANTIBODY RESPONSE TO BACTERIOPHAGE φX174 IN RATS†

	Rat number	*Number of small lymphocytes transferred* ($\times 10^6$)	*Challenge I.V.* 10^{10}φX *(days after primary immunization)*	*Serum antibody (K) (days after challenge)* 7	14	21
Primary response 10^{11} φX	1	—	—	1·1	3·1	8·2
	2			0·6	1·9	25
Secondary response	1	—	26	146	203	180
	2			605	632	310
Small lymphocytes from primary donor I.V. to 500 rad recipients. Recipients challenged	3	430	71	121	253	104
	4	330		101	184	50
	5	260		610	521	—

† Uhr and Gowans (1966).

hours after they had received 500 rads of whole body X-irradiation. The recipients were then challenged with a single intravenous dose of 10^{10} PFU. Table I shows that a substantial "secondary" response was obtained in the recipient animals. These preliminary results suggest very strongly that one of the cell types which responds to secondary challenge is the small lymphocyte. There is no inconsistency between this finding and the observation that secondary responses in rats to sheep erythrocytes (Gowans and McGregor, 1963), and to tetanus toxoid (McGregor and Gowans, 1963), are insensitive to prior lympho-

cyte depletion by chronic drainage of cells from the thoracic duct. In such lymphocyte-depleted animals sufficient cells presumably remain in the lymphoid tissue to mediate the response. These sessile cells may either be representatives of a dividing cell line, perhaps aggregated in the form of germinal centres, or they may be a population of small lymphocytes of the kind noted by Miller (1964), which are not part of the recirculating pool, and which consequently cannot be extracted from the animal by thoracic-duct drainage. The origin of the "committed" small lymphocytes which it is suggested may mediate long-term immunological memory is not known but the simplest notion would be that they arise from the same precursors as produce plasma cells.

ANTIBODY FORMATION IN THE ISOLATED PERFUSED SPLEEN

It can be argued that the two functional classes of small lymphocytes which have been postulated from experiments on transplantation immunity may also be involved in classical antibody responses. Some evidence that "committed" small lymphocytes may mediate long-term secondary responses has just been considered; there is other evidence that primary responses to sheep erythrocytes and to tetanus toxoid in rats may also be initiated by small lymphocytes (McGregor and Gowans, 1963; Gowans and McGregor, 1963). These demonstrations that small lymphocytes from the thoracic duct of rats possess immunological activity raise the question of the immunological significance of the process of lymphocyte recirculation (Gowans *et al.*, 1962; Hall and Morris, 1964). In the present experiments this problem has been studied, in relation to the primary response to sheep erythrocytes, employing the isolated perfused spleen of the rat. The aim was to discover if the magnitude of the primary response was determined simply by the number of lymphocytes in the spleen or whether it was dependent in any way upon the concentration of lymphocytes in the perfusate.

The isolated spleens of normal (non-immune) adult rats of a highly inbred strain were perfused by means of the apparatus illustrated in Fig. 7. The spleen, with its artery and vein cannulated, was placed under paraffin oil in an organ chamber held at 37°C. It was perfused with heparinized rat blood, the haematocrit

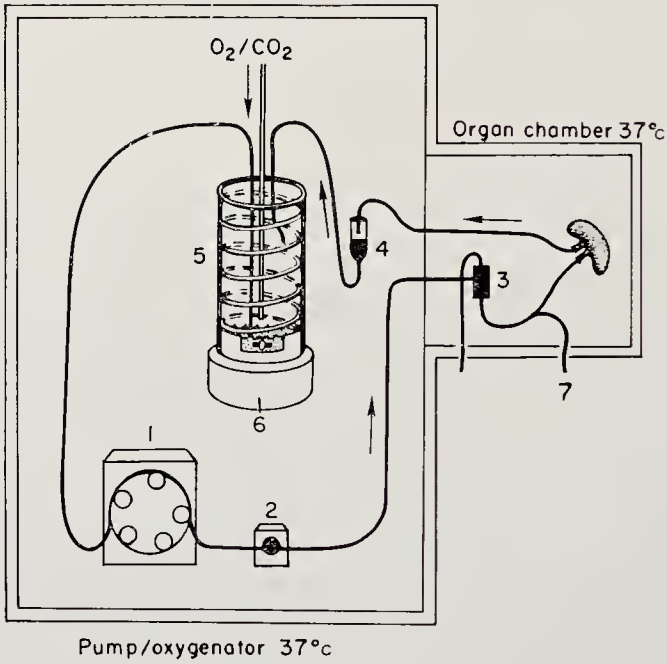


FIG. 7. Apparatus for perfusing isolated rat spleen.
(1) Roller pump. (2) Filter. (3) Bubble trap. (4) Dripper. (5) Oxygenator. (6) Magnetic stirrer. (7) Lead to manometer.

of which had been reduced by mixing it with an approximately equal volume of rat plasma. The venous effluent from the spleen was oxygenated as it passed down a spiral platform in a cylindrical perspex chamber through which an O_2/CO_2 mixture was flowing. The oxygenated blood, which collected in the base of this chamber was mixed by a slowly revolving magnetic stirrer and then pumped by means of a roller pump through a filter and a

bubble trap into the splenic artery. By this means, spleens could be perfused at the rate of approximately 1 ml./min. for periods up to 10 hours at a pressure of about 40 mm. of mercury. To initiate an antibody response, a standard dose of 10^8 sheep erythrocytes was added to the perfusate. The perfusate was also

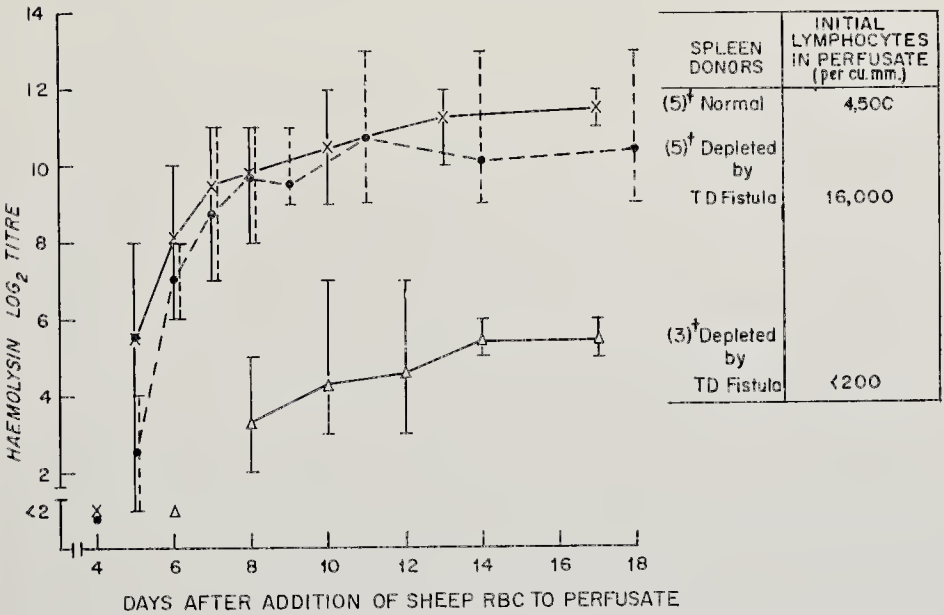


FIG. 8. Mean and range of haemolysin responses in X-irradiated rats after implanting fragments of perfused spleens. ×——× = response of normal spleens after perfusion with blood containing a normal number of small lymphocytes. Response of lymphocyte-depleted spleens (△——△) was restored to normal (●---●) by perfusing them *in vitro* with lymphocyte-enriched blood.
† = number of experiments in each group.
TD = thoracic duct.

supplemented with syngeneic thoracic-duct lymphocytes to give an initial concentration in the perfusate of 4500 cells/cu. mm. Normal spleens were perfused for 3 hours following the addition of lymphocytes and antigen and, at the end of this period, were divided into about 30 small fragments that were implanted into the peritoneal cavity of syngeneic recipients which had received 500 rads of whole body X-irradiation 24 hours previously. Fig. 8

shows that a substantial haemolysin response was obtained in a series of five such recipients. Fig. 8 also shows that spleens from rats which had been depleted of lymphocytes *in vivo*, by chronic drainage of cells from a thoracic-duct fistula, produced very little haemolysin in X-irradiated hosts after they had been perfused *in vitro* for 3 hours with antigen and blood containing less than 200 lymphocytes/cu. mm. However, the response of such lymphocyte-depleted spleens could be restored to normal by perfusing them with blood supplemented with thoracic-duct lymphocytes to a concentration of 16,000 cells/cu. mm. These experiments on isolated spleens are the counterpart of the demonstration in the whole animal that chronic drainage of lymphocytes from a thoracic-dust fistula profoundly depresses the haemolysin response of rats, but that the response can be restored to normal by intravenous injections of normal syngeneic small lymphocytes (McGregor and Gowans, 1963).

In further experiments, spleens from normal donors were perfused for 3 hours with "lymphocyte-free" blood (less than 200 lymphocytes/cu. mm.) to which the standard dose of sheep red cells had been added at the beginning of the experiment. When such spleens were transferred to X-irradiated recipients it was found that the haemolysin response was considerably lower than that obtained with identical spleens which had been perfused for the same period with blood containing 4500 lymphocytes/cm. mm. (Fig. 9). The obvious interpretation of this experiment was that the low haemolysin titres were a reflection of the lymphocyte-depleted state of the organ, particularly since it was found that perfusion with lymphocyte-free blood had resulted in the emigration of large numbers of lymphocytes from the spleen. An attempt was therefore made to accentuate this effect by perfusing normal spleens with lymphocyte-free blood for a number of hours before the addition of antigen, the aim being to abstract as many lymphocytes as possible from the spleen into the perfusate before antigenic stimulation. Fig. 9

Duration of perfusion (hours)	Time after start of perfusion when antigen added	Lymphocytes / cu. mm. in perfusate	
		Initially	at time of antigen addition
(3)† 6	5	<200	3500
(4)† 3	0	<200	<200

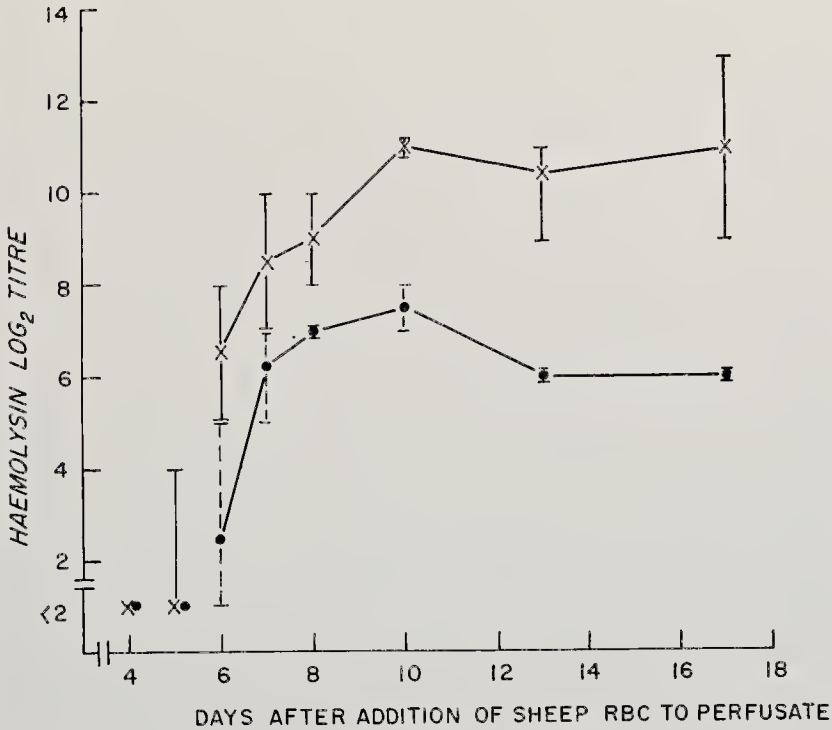


FIG. 9. Mean and range of haemolysin responses in X-irradiated rats after implanting fragments of perfused spleens. In both groups of experiments the perfusate contained initially <200 lymphocytes/cu. mm. In one group, antigen was added at the start of perfusion (●——● and lower line of table); in the other, the addition of antigen was delayed for 5 hours (× ——× and upper line of table).
†= number of experiments in each group.

shows the haemolysin responses obtained from three normal spleens which were perfused for 5 hours with blood containing initially less than 200 lymphocytes/cu. mm.; the standard dose of

antigen was added after 5 hours and the perfusion continued for a further 1 hour. Contrary to all expectations, the response of these spleens, far from being further depressed, was in fact restored to normal.

An explanation which covers the results of all the splenic perfusion experiments is that the magnitude of the haemolysin response depends not on the number of lymphocytes within the spleen at the time of antigen administration but upon the concentration of lymphocytes in the blood perfusing it. It can be seen from Fig. 9 that after perfusion for 5 hours with blood which was initially lymphocyte-free, considerable numbers of lymphocytes had entered the perfusate from the spleen to build up and maintain a concentration of about 3500 cells/cu. mm. Further, in experiments with radioactively-labelled lymphocytes, it was found that the rate of transfer of lymphocytes from the perfusate into the spleen was directly proportional to their concentration in the perfusate. These results suggest that, to initiate a maximal antibody response to sheep red cells, a traffic of lymphocytes must be passing through the spleen at the time of antigenic stimulation. One possibility is that lymphocytes must make contact with the relevant antigen-containing macrophages within the spleen so that antibody formation may be induced, and that to ensure a sufficient number of "hits" a considerable number of lymphocytes must pass through the macrophage-containing compartment.

This notion cannot yet be formulated in precise anatomical terms. In both the intact and the isolated spleen, labelled lymphocytes which are introduced into the blood achieve their highest concentration in the zone of the white pulp which immediately surrounds the central arteriole. Whether their route from the blood into this zone takes lymphocytes through areas rich in macrophages has yet to be established. In any event it is not certain which anatomical class of macrophages, if any, is essential for the processes which lead to antibody formation.

MACROPHAGE-LYMPHOCYTE INTERACTION *in vitro*

The experiments described in this section aimed at determining whether phagocytosis of antigen by macrophages was in fact an essential preliminary step in the induction of antibody formation by lymphocytes.

The work of Fishman and Adler (1963) has strengthened the idea that macrophages may play an essential role in the induction of antibody formation: they found that a ribonuclease-sensitive material extracted from antigen-containing macrophages could stimulate the formation of small quantities of antibody by lymph-node cells. Subsequent work by Askonas and Rhodes (1965) and Friedman, Stavitsky and Solomon (1965) makes it very likely that the extracted material is a complex of antigen with RNA. This reservation does not affect the possibility that the processing of antigen by macrophages may be a necessary first step during induction. Anatomical evidence of adhesion between lymphocytes and macrophages in immunized lymph nodes is further support for this possibility (Sharp and Burwell, 1960; Schoenberg *et al.*, 1964). However, there has as yet been no demonstration that *living* macrophages which have ingested antigen are able to prime lymphoid cells for antibody formation *in vitro* under conditions where lymphoid cells cannot be stimulated by native antigen directly. The scheme of an experiment which was designed to investigate this point, employing sheep red cells as antigen, is shown in Fig. 10.

In each experiment about 10^8 lysed sheep red cells were injected intraperitoneally into each of 10 rats killed with ether; 20 minutes later the macrophages were harvested in peritoneal washings. The macrophages were cultivated in glass vessels in a serum-free medium to which thoracic-duct lymphocytes from normal rats were added. The mixture of lymphocytes and macrophages was incubated for 3–6 hours at 37°C during which time most of the macrophages adhered to the surface of the glass. At the end of

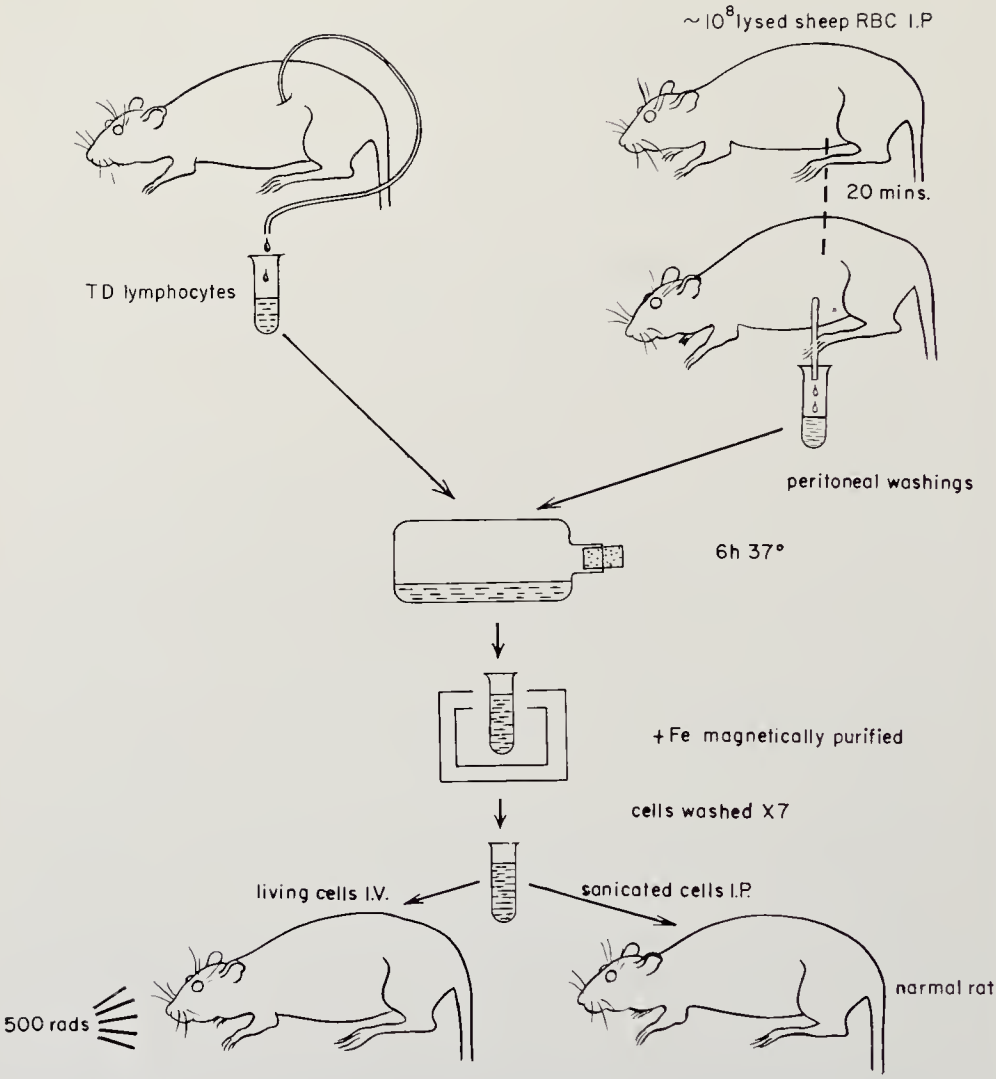


FIG. 10. Scheme of experiment to initiate antibody production by exposing lymphocytes *in vitro* to macrophages laden with sheep erythrocytes (see text).

the period of incubation the non-adherent cells were poured from the bottle and mixed with a carbonyl-iron preparation; this allowed any phagocytic cells in the suspension to be removed magnetically and rejected. The remaining cells were then washed seven times and divided into two equal portions. One portion

was ultrasonically disintegrated and injected intraperitoneally into a normal rat to determine if any free antigen remained in the inoculum. The other portion, consisting almost exclusively of living lymphocytes, was injected intravenously into a rat which had received 500 rads of whole body X-irradiation 24 hours previously. Table II records a typical experiment in which the "free antigen" control was negative but where a substantial formation of haemolysin occurred in the X-irradiated recipient. A similar experiment in which thoracic-duct lymphocytes were incubated *in vitro* with lysed sheep red cells, but from which macrophages were excluded, yielded negative results both for the carry over of free antigen and for the adoptive formation of antibody in the X-irradiated recipient. In this experiment the period of incubation was only 3 hours but other experiments, of which one is noted in Table II, have shown that this period is sufficient for the induction of haemolysin formation in a system containing both macrophages and lymphocytes. These experiments suggest that the ingestion of sheep erythrocytes by macrophages is an essential preliminary step in the initiation of haemolysin formation by lymphocytes. No attempt was made to determine the precise identity of the lymphocytes in which antibody formation was induced but previous work (Gowans and McGregor, 1963) makes the small lymphocyte the most likely candidate.

DISCUSSION

Experiments on a graft-versus-host reaction have shown that, under the impact of mouse antigens, rat small lymphocytes enlarge, start dividing and generate more small lymphocytes. The suggestion that this may indicate the existence of two classes of small lymphocytes—uncommitted and committed—begs important questions about the nature of immunological commitment. For example, even assuming an elective theory of antibody formation, immunization may either increase, by cell division,

TABLE II

HAEMOLYSIN RESPONSE IN X-IRRADIATED RATS INJECTED WITH LYMPHOCYTES WHICH HAD BEEN INCUBATED *in vitro* WITH ANTIGEN-LADEN MACROPHAGES OR WITH ANTIGEN ALONE

Incubation mixture			*Recovered lymphocytes*			*Recipients*								
Composition	*Number of leucocytes*	*37°C for (time in hours)*	*Treatment before washing*	*Number of washes*	*Treatment before injection*	*Status*	*Log_2 haemolysin titre: days after I.V. lymphocytes*							
							3	4	5	6	7	8	9	10
10^8 lysed RBC + TDL	6×10^8	3	—	5	—	500 rads	<2	→						
					sonicated	normal	<2	→						
Macrophages† + TDL	2×10^8 / 7×10^8	3	—	9	—	500 rads	<2	<2	5	6	6	8	6	4
Macrophages† + TDL	2×10^8 / 7×10^8	6	+Fe magnetic purification	7	—	500 rads	<2	<2	<2	3	5	6	7	4
					sonicated	normal	<2	→						

† Macrophages from rats given 10^8 lysed sheep erythrocytes intraperitoneally.
TDL = Thoracic-duct lymphocytes.

the number of lymphocytes already possessing specific reactivity or it may switch on a mechanism which was latent. In the former case there would be no qualitative difference between individual committed and uncommitted cells. Following immunization with skin grafts, *populations* of lymphocytes have been shown to acquire specific reactivity towards target tissue *in vitro* which was not demonstrable before immunization (Wilson, 1963); but this does not necessarily mean that a new class of cell has been created.

Accounts have been given of two other experiments: one indicates the importance of circulating lymphocytes in the formation of haemolysin to sheep erythrocytes by the isolated perfused spleen, and the other shows that macrophages may be essential for the inductive process. The observations reinforce the view that, at a cellular level, lymphoid organs cannot be regarded as static structures in which cells have the kind of permanent anatomical relationship to each other which is caricatured in histological sections. It seems likely that, during the induction of antibody formation, a recruitment or selection of cells from the recirculating pool of lymphocytes takes place within stimulated lymphoid tissue and this ensures that sufficient cells become eligible for induction. Such a process would counteract two factors which may operate to restrict the number of cells induced. First, during primary responses, it is possible that only a small proportion of lymphocytes are potentially able to react to a particular antigen (in the sense embodied in elective theories of immunity) and that a large population of cells must consequently be made available for selection. The observations on the carriage of immunological memory to bacteriophage φX 174 by small lymphocytes suggest that a selection of potentially reactive cells may also occur during secondary responses. Second, the mere presence of competent lymphocytes within a regionally stimulated lymphoid organ may not necessarily ensure that antibody formation is induced in these cells: they may have to make intimate

contact with macrophages. The requirements imposed by these two factors would be met if a large traffic of lymphocytes passed through a lymphoid organ and would provide a *raison d'etre* for the process of lymphocyte recirculation.

SUMMARY

A number of experiments are described which bear on the problems of the origin and function of lymphocytes, and on the immunological significance of lymphocyte recirculation.

(1) The large pyroninophilic cells which evolve from rat small lymphocytes during a graft-versus-host reaction in irradiated mice divide to produce more small lymphocytes. These small lymphocytes were identified in the thoracic-duct lymph of rats which had been injected with cell suspensions prepared from the chimaeric mouse spleens.

(2) The idea that small lymphocytes may carry immunological memory was supported by experiments in which X-irradiated rats responded in a secondary manner to a first dose of bacteriophage φX 174 after they had been injected with small lymphocytes from primarily immunized donors.

(3) Studies on the haemolysin response to sheep red cells were carried out on the isolated perfused spleen of the rat. The magnitude of the response was found to depend upon the concentration of lymphocytes in the perfusate at the time antigen was added to it.

(4) Haemolysin formation to sheep red cells could be induced in lymphocytes by confronting them *in vitro* with living antigen-laden macrophages, but not by treating them with antigen directly.

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DISCUSSION

Burnet: Professor Gowans, as well as this mobility that you have demonstrated between the blood and lymph circulations, do you think that within the lymphoid tissues themselves there is active mobility of the component lymphocytes? If this were so it might help greatly in your macrophage-lymphocyte contact.

Gowans: Yes, I think this is quite likely. Lymphocytes are motile and it is possible that this property may lead to cellular mixing within the nodes; the lymph nodes of the living animal must be rather like a bag of squirming worms!

Burnet: If this is true the bag of worms might be employed to make the best possible use of macrophages, particularly the rather fixed dendritic macrophages of Nossal and associates (Nossal, G. J. V., Ada, G. I., and Austin, C. M. [1964]. *Aust. J. exp. Biol. med. Sci.*, **42**, 311). Contact could be maintained between the macrophages and this bag of worms whose contents are being constantly recruited from the circulation. Is this your idea, Professor Gowans?

Gowans: Yes. The maximum number of macrophage-lymphocyte contacts could be assisted by a process of internal cellular mixing as you suggest, and also by traffic of lymphocytes through the lymph node. I am attracted to the idea that such a traffic may be important partly because of W. L. Ford's finding (this volume, p. 67) that the concentration of circulating lymphocytes influences the immunological performance of the isolated perfused spleen, and also through the ingenious experiments of J. C. Hall and B. Morris (1964. *Lancet*, **1**, 1077) which showed that, in sheep, a heavy dose of local X-irradiation to the popliteal lymph node scarcely affected its ability to produce antibody.

Clark: In our thinking about the compartment in which the lymphocyte comes into contact with the right sort of antigen product, I wonder if we are overlooking something if we do not suspect that this compartment might be the peripheral blood itself, as this is the one place where lymphocytes from all sources meet. Professor Gowans, do you think that the macrophages, if they are necessary for processing antigen, might release some product into the blood which the lymphocytes can meet there more easily than in the crowded and secluded quarters of the lymphoid tissues themselves?

Gowans: There are a number of arguments against the idea that lymphocytes interact with antigen in the blood. No one has been able to induce antibody formation in lymphocytes by confronting them with antigen directly; the co-operation of macrophages appears to be necessary as shown by the experiments of M. Fishman (1961. *J. exp. Med.*, **114**, 837) and P. J. McCullagh (this volume, p. 73). The reports that human peripheral-blood lymphocytes from appropriate donors will enlarge and produce antibody when cultivated *in vitro* with the corresponding antigens cannot, in my view, be accepted on the present evidence. Anatomical observations on macrophage-lymphocyte associations in lymph nodes suggest that close contact between these

cell types (Sharp, J. A., and Burwell, R. G. [1960]. *Nature, Lond.*, **188** 474) to the point of actual cytoplasmic bridging (Schoenberg M.D., *et al.* [1964]. *Science*, **143**, 964) may have a functional significance in immune reactions. Finally, studies on the localization of antigens, at least of particulate antigens, in the whole animal point to the lymph nodes and spleen as the main sites for antigen-lymphocyte interactions.

Clark: One of my reasons for mentioning this is that it seems to me important to visualize some mechanism for recruiting lymphocytes from distant sites to the vicinity of a local deposit of antigen.

Gowans: I agree. The process of lymphocyte recirculation would provide a nice mechanism for recruiting extra lymphocytes, or a special class of lymphocyte, from the blood (and thus from the general lymphoid mass in the animal) into regionally stimulated lymph nodes. It is also possible that sensitization may occur peripherally in the tissues. For example, lymphocytes might emigrate from the blood vessels into a subcutaneous area where antigen had been deposited and interact locally with macrophage-processed material. After induction the lymphocytes could travel by way of the afferent lymph to the regional nodes. Observations on the cellular composition of afferent lymph draining a subcutaneous antigen depot have shown that a process of this kind may occur (Hall, J. C., and Morris, B. [1963]. *Q. Jl exp. Physiol.*, **48**, 235). There is also some evidence that peripheral sensitization may occur when lymphocytes traverse organ and tissue homotransplants (Strober, S., and Gowans, J. L., [1965]. *J. exp. Med.*, **122**, 347) but no role has yet been assigned to macrophages in the processing of antigens for the induction of transplantation immunity.

Šterzl: Professor Gowans, what is the difference between your present and your previous experiments? I think that in the past you described the cells with a role in the secondary response as sessile dividing cells—large cells—that arise in the regional lymphoid tissue as a result of primary immunization, whereas it is the small circulating lymphocytes that play a predominant part in the primary response (Gowans, J. L., and McGregor, D. D. [1963]. *In* Immunopathology, Proc. III Int. Symp., p. 89, ed. Grabar, P., and Miescher, P. A., Basel: Schwabe.

Gowans: Yes. Some of our previous observations showed that primary, but not secondary, responses in rats were sensitive to lympho-

cyte depletion (McGregor, D. D., and Gowans, J. L. [1963]. *J. exp. Med.*, **117**, 303). This means that the cells which remained behind in the depleted lymphoid tissue could still mediate the secondary response. The nature of these "fixed" cells was not determined. One possibility was that they were large dividing lymphocytes of the kind suggested by G. J. V. Nossal and O. Mäkelä (1962. *J. exp. Med.*, **115**, 209) but this is now by no means certain (Cohen, E. P., and Talmage, D. W. [1965]. *J. exp. Med.*, **121**, 125). The work which J. W. Uhr and I did (to be published) in New York on the response of rats to φX bacteriophage makes the small lymphocyte an additional candidate.

Kaplan: There is good evidence that the lymphocyte acquires its immunological capacities as it matures. Professor Gowans, is there any similar evidence that the immunological capacities of the macrophage also evolve, and that it learns with age to do what you have just described?

Gowans: We have not tested macrophages from animals of varying age in our *in vitro* system. It may be of some interest, although this does not bear directly on your question, that, in preliminary studies, we have found macrophages from immunologically tolerant donors to be effective when cultivated *in vitro* with normal lymphocytes, but that an intriguing complication has arisen in testing the performance of normal macrophages with lymphocytes from tolerant donors. In a large number of tests, lymphocytes from donors tolerant of sheep erythrocytes have always failed to restore reactivity to a subsequent injection of erythrocytes in heavily X-irradiated rats; but in a number of instances some tolerant lymphocytes have yielded small amounts of antibody in irradiated hosts after incubation *in vitro* with normal antigen-containing macrophages. In other words, some unidentified manœuvre in the *in vitro* system is causing tolerance to break in a proportion of the experiments.

Rowley: We have some information that may be relevant to Dr. Kaplan's question. We have been studying the properties of macrophages from mice and rats at varying stages of embryonic development. The macrophages from embryonic animals are fully capable of phagocytosis but incapable of digesting bacteria as compared with macrophages from adult animals.

Gowans: Is there a deficiency of any serum factor?

Rowley: No, in these animals the bacteria were opsonized and antibody-treated.

Strauss: With reference to Professor Gowans' concept of the relationship of lymphocytes and macrophages, I would like to ask Professor Good if there are any experiments of nature in which there is impairment in the macrophage system with a normal lymphocyte complex. If there are, it might provide confirmatory evidence for Professor Gowans' theory.

Good: There are two very interesting diseases where this possibility is suggested. In Aldrich's syndrome (Krivit, W., and Good, R. A. [1959]. *Am. J. Dis. Child.*, **97**, 137; Cooper, M. D., *et al.* [1964]. *J. Lab. clin. Med.*, **64**, 849; St. Geme, J. W., *et al.* [1965]. *New Engl. J. Med.*, **273**, 229) patients have great difficulty in developing responses to isohaemagglutinins although they are responsive to many other antigens. These patients seem to have difficulty on the afferent side in dealing with certain types of antigen whereas their efferent side—their γ-globulins —are normal. The second such disease, lipochrome histiocytosis, has been described by Dennis Ford and others (Ford, D. K., *et al.* [1962]. *Am. J. Med.*, **33**, 478; Landing, B. H., and Shirkey, H. S. [1957]. *Pediatrics*, **20**, 431). In this syndrome an increased susceptibility to infection is associated with lipochrome histiocytosis of the macrophages. Affected individuals have some depression of their ability to resist infection. This subject needs a great deal more investigation; not enough attention has been paid clinically to the input or afferent side of the immune response.

Dameshek: Another experiment of nature which links up with Professor Good's comments, and also with Dr. Clark's question about the possible site of action of the lymphocytes, concerns the body's reaction to acute pyogenic infections. One of the features of this reaction is that the blood lymphocyte count drops precipitously. I think it is not impossible that the lymphocytes leave the blood and migrate to a site where extra numbers of these cells are needed. This reaction to pyogenic infections might be described as a general and very common experiment of nature.

Woodruff: Professor Gowans, do you accept the view for which Dr. Howard and co-workers produced some evidence at a CNRS colloquium in September, 1964, that small lymphocytes may give rise to

macrophages (Howard, J. G., *et al.* [1965]. *In* La Greffe des Cellules Hématopoietiques Allogéniques, p. 95. CNRS: Paris)?

Gowans: I have seen Howard's evidence and find it very convincing.

Woodruff: If you do accept this evidence it raises the possibility that the macrophage inherits some kind of commitment from its lymphocyte ancestor.

Gowans: This is a complex question, for it may be that macrophages can have different origins depending upon the stimulus which provoked their appearance. A. Volkman and I (1965. *Br. J. exp. Path.*, **46**, 50, 62) are convinced that the macrophages which accumulate on Rebuck skin windows in rats are not derived from lymphocytes but probably from a rapidly dividing precursor in the bone marrow. A similar conclusion about the non-lymphoid origin of macrophages in granuloma pouches in rats has been reached by W. G. Spector, M. N-I. Walters and D. A. Willoughby (1965, *J. Path. Bact.*, **90**, 181). On the other hand, J. G. Howard (personal communication) has shown with a chromosome marker that dividing phagocytic cells in the liver of mice undergoing a graft-versus-host reaction were derived from a cell type in the inoculum of allogeneic thoracic-duct lymphocytes. Thus, in a graft-versus-host reaction, macrophages in the liver (presumably Kupffer cells) were derived from lymphoid precursors, possibly small lymphocytes.

Woodruff: Professor Gowans, in your presentation you quoted some figures on the life-span of the lymphocyte which seemed rather high. If you are thinking of a mean life-span of human lymphocytes of ten or fifteen years, one does wonder how the body finds room for all the new lymphocytes.

Gowans: I was talking about the *potential* life-span of lymphocytes; these figures do not mean that all lymphocytes have such a long life. Indeed, in rats a separate class of small lymphocytes appears to exist which has a short life, measured in days only (Caffrey, R. W., Rieke, W. O., and Everett, N. B. [1962]. *Acta haemat.*, **28**, 145).

Boyden: Professor Gowans, in the experiment you described in which the results suggested that the macrophages play an essential role in the lymphocyte's response to antigen, why did you give your initial injection of antigen intraperitoneally? This would have been a cleaner experiment if the macrophages could be shown to phagocytose well

in vitro. In the peritoneal cavity unexpected complications might occur and influence the reaction.

Gowans: We did this because we found it difficult to persuade macrophages to phagocytose sheep red cells *in vitro*.

Boyden: Perhaps the best time for phagocytosis of the red cells would have been after the macrophages had spread out on the glass surface.

Gowans: Feeding lysed sheep red cells to the macrophages *in vivo* helped to get over another difficulty in these experiments, namely, that of obtaining presumptively sensitized lymphocytes which were uncontaminated with free antigen.

Boyden: The control would have taken care of that.

Gowans: Yes, the control eventually did, but this involved a magnetic clearance of the residual macrophages and repeated washing of the lymphocytes in order to yield a population of cells which, after sonication, would not actively immunize a normal rat.

DIFFERENTIATION AND IMMUNOLOGICAL COMPETENCE OF CLONED CELL POPULATIONS OF LYMPHOID ORIGIN

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Two distinct cell systems were claimed to represent the primordium of lymphoid cells in mammals. During the early stages of embryogenesis lymphocytes were demonstrated to differentiate from the epithelial component of the thymus rudiment (Auerbach, 1961). Once formed within the embryonic thymus, they appear to migrate out, colonize the spleen and lymph nodes and are believed, although not directly shown, to constitute the immunologically competent cells of the lymphoid system (Auerbach, 1963; Auerbach, 1964*a*, *b*). At later stages of ontogenesis the thymus, at least under the experimental conditions studied, was found to induce differentiation of lymphocytes from cells of extrinsic origin (Levey, Trainin and Law, 1963; Levey *et al.*, 1963; Osoba and Miller, 1963; Globerson and Feldman, 1964). Cells of bone marrow origin represent the precursors which, in the irradiated organism, may differentiate to immunologically competent lymphocytes in response to an inductive stimulus of the thymus (Feldman and Globerson, 1964). Whether, under normal conditions of neonatal or adult differentiation, cells of bone marrow or another extrinsic tissue serve as targets for the inductive lymphopoietic stimulus of the thymus remains an open question.

Applying morphological criteria lymphoid cells are subdivided into a limited number of categories. Yet, in testing their functional properties, a striking heterogeneity is manifested in the production of different antibodies by different cells. It has therefore been suggested by Burnet (1959) and Lederberg (1959) that the cell population of the lymphoid system is, in fact, heterogeneous with respect to genes coding for γ-globulins, each cell carrying genes for different antibodies. An antigen, according to Burnet's most stimulating concept, will trigger the replication of only that cell (or cells) which carries the genes for its corresponding antibody, thus producing a clone of cells reactive to that specific antigen only. Each cell within the lymphoid population of a single organism is thus restricted in its potency to respond to antigenic stimuli. On the other hand, it was conceived by others that each lymphoid cell is *a priori* pluripotential, that is, capable of forming antibodies to any given antigen. The concept of pluripotentiality may envisage either an instructive mechanism of antibody production (Breinl and Haurowitz, 1930; Pauling, 1940; Karush, 1958; Haurowitz, 1965), or a state in which every antibody is determined by a specific gene but the entire spectrum of such genes is located within the genome of each immunologically competent cell (Szilard, 1960). Such pluripotential cells, once stimulated by a particular antigen, may not respond to other antigens. Thus, the demonstration that in rats immunized with two different salmonella antigens each cell produced antibody to only one antigen at a time (Mäkelä and Nossal, 1961; Nossal and Mäkelä, 1962) need not mean that these cells were predetermined to produce only the antibody they were found to have produced.

The present report outlines an attempt to study whether lymphoid cells are pluripotential for antibody production or whether each cell is predetermined for one specific antibody. This work was carried out by cloning cells of lymph node origin before an antigenic stimulation and then testing the

immunological reactivity of individual clones to a given antigen. If each cell within a cloned population mirrors the genetic pattern of its original clone-forming cell, that is, if mutations did not alter the genetic structure of the cloned population, then the clonal selection concept of antibody production would predict that, of randomly chosen clones, practically none would react to a randomly chosen antigen. On the other hand, the "pluripotential" hypothesis would predict that each clone would be reactive to any antigen.

To produce clones of lymphoid origin, we adapted to lymphoid cells the intrasplenic cloning method of Till and McCulloch (1961). Our approach (Mekori, Chieco-Bianchi and Feldman, 1965) was based on the expected blastogenic effect of phytohaemagglutinin (PHA) on small lymphocytes (Mackinney, Stohlman and Brecher, 1962; Berman and Stulberg, 1962; Schrek and Rabinowitz, 1963). Mice were treated each with 0·3 ml. of PHA-m (Difco), and three days later the animals were sacrificed and spleen cell suspensions were made from pooled inguinal, brachial and mesenteric lymph nodes of individual donors. The suspended lymph node cells were then injected into lethally irradiated (850 r.) syngeneic mice. The inoculated cells gave rise to intrasplenic noduli which were excised individually for further treatment 12 days following cell inoculation. There was a linear relationship between the cell dose inoculated and the incidence of spleen nodules, indicating that a colony can be produced by a single cell (Mekori, Chieco-Bianchi and Feldman, 1965). This was further substantiated by demonstrating that the incidence of spleen colonies in different experiments fitted the hypothesis of a Poisson distribution (Feldman and Mekori, 1966). It was therefore concluded that a spleen nodule represents a cloned population of cells resulting from the replication of a single cell of lymph node origin.

An analysis of the cell types represented within the spleen noduli has shown that cells of two distinct lines constitute the population of each colony. Peroxidase stain revealed the occur-

rence of cells of the erythroid line mixed with many blast-type cells and cells of the lymphoid line. The latter showed a relatively large number of pyroninophilic cells among which mature plasma cells were found. This appears to indicate that the clone-forming cell, which could have undergone a blastogenic transformation due to PHA, gave rise to cells of both the lymphoid and erythroid lines.

The lymph-node-originating clones were then tested for their immunological competence (Feldman and Mekori, 1966). In testing the capacity of cloned cell populations of lymph node origin to produce antibody to shigella antigen, lymph node cells from female C57BL mice were cloned in X-irradiated syngeneic mice. Seven days following cell inoculation (5×10^7 cells per recipient) test animals (donor group A) and irradiated control mice which were not inoculated with lymph node cells (donor group B) were immunized with antigen (intraperitoneal injection, 0·2 ml. of 0·1 per cent alcohol-killed *Shigella dysenteriae*,† courtesy of Dr. T. N. Harris). Five days later, that is on day 12 following cell inoculation, the animals were killed and the spleens excised. Cell suspensions of 8 individual noduli of donor group A were now injected into 8 X-irradiated syngeneic mice (female, C57BL). A second group of 7 irradiated mice were each injected with 8×10^6 spleen cells of donor group B. A third and fourth group of irradiated animals were inoculated with bone-marrow cells from normal animals which were immunized with shigella antigen similarly to groups A and B; the third group received 5×10^5 cells and the fourth, 1×10^6. Five days later, the animals of all four groups were immunized with shigella antigen and then tested for agglutinin titre on day 11 following cell inoculation. The results (Table I) indicate that the only group which produced antibody to shigella antigen was the one in which the animals were inoculated with clones of lymph-node origin. Five out of

† The antigen from *Shigella dysenteriae* is referred to throughout this paper as shigella antigen.

TABLE I

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS

Group number	*Treatment:** *X-irradiated mice inoculated with*	*Number of reacting animals*	*Number of surviving animals*	*Per cent animals reacting*	*Titre $\log_2$ produced by animals* — *Animals' numbers:* 1	2	3	4	5	6	*Mean titre $\log_2$*
1	Immunized, lymphoid clones (from donor group A)	5	6	83	10	9	0	7	5	7	6·33
2	8×10^6 spleen cells (from donor group B)†	0	6	0							0
3	5×10^5 bone-marrow cells‡	0	14	0							0
4	10^6 bone-marrow cells‡	0	17	0							0

* All animals were injected with shigella antigen (see text) 5 days following cell inoculation.
† From immunized, X-irradiated mice.
‡ From normal, immunized mice.
For full explanation, see text.

six animals, each of which had been treated with cells of a different single clone, showed agglutinating antibodies with a mean log titre of 6·33.

In a second experiment a comparison was made between the antibody production of cloned cells which were immunized only once in the primary recipient and that of clones immunized in the primary and in the secondary recipients. Having found in the previous experiment that cell populations of lymph-node-originating clones did produce antibody to shigella antigen whereas bone-marrow cells, even from immunized animals, did not, attempts were now made to improve the survival of the secondary X-irradiated recipients by injecting bone-marrow cells in addition to populations of the cloned cells. To see if the inoculated bone-marrow cells were participating in the immune response, control X-irradiated animals were injected with bone marrow from immunized donors, then further challenged with shigella antigen. The following experiment was carried out: Donor group A: 44, female $(C3H \times C57BL)F_1$ irradiated mice were each injected with 4×10^7 lymph-node cells from PHA-treated donors. Donor group B: 23, female $(C3H \times C57BL)F_1$ animals were irradiated controls. Six days following exposure the two groups and, in addition, 10 normal animals (donor group C) were immunized with shigella. On day eleven, 68 secondary recipients were X-irradiated and divided into 5 groups: *Group* 1: 18 animals were injected with a cell population of a single clone of donor group A and 1×10^6 syngeneic bone-marrow cells from normal donors. *Group* 2: 9 animals were each injected with 8×10^6 spleen cells from donor group B and 1×10^6 bone-marrow cells. *Group* 3: 20 animals were each injected with 1×10^6 bone-marrow cells. *Group* 4: 11 animals were each injected with 2×10^6 bone-marrow cells from donor group C. *Group* 5: 10 control irradiated animals did not receive any cell injection. On day 16, 9 animals of group 1 (designated group 1A) were immunized with shigella antigen. On day 21, all animals

TABLE II

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS

Group number	Treatment: X-irradiated mice inoculated with:	Number of reacting animals	Number of surviving animals	Per cent animals reacting	Titre $\log_2$ produced by animals — Animals' numbers 1	2	3	4	5	6	7	8	9	10	11	12	Mean titre $\log_2$
1	Immunized, lymphoid clones; 10^6 bone-marrow cells	8	8	100	5	5	6	7	5	5	3	2					4·75
1A	Immunized, lymphoid clones; 10^6 bone-marrow cells; second immunization	9	9	100	10	9	6	7	5	8	5	10	10				7·66
2	8×10^6 spleen cells;† 10^6 bone-marrow cells; shigella antigen	0	8	0													0
3	10^6 bone-marrow cells;‡ shigella antigen	0	18	0													0
4	2×10^6 bone-marrow cells;‡ shigella antigen	0	10	0													0
5	Shigella antigen	0	3	0													0
6	Normal, non-irradiated control; shigella antigen	12	12	100	10	10	9	10	4	5	6	9	10	10	7	7	8·08

† From immunized, X-irradiated donors.
‡ From immunized, normal donors.
For full explanation, see text.

were bled and the sera from the individual mice were tested for agglutinin to shigella. The results (Table II) showed: (*a*) that each of the lymphoid clones tested produced antibodies to shigella antigen whereas none of the controls produced any detectable antibody; and (*b*) that animals with cloned cell populations which were challenged twice with the antigen (group 1A), rather than once (group 1) showed a higher titre of agglutinating antibodies (mean titre $\log_2$ value 7·7 to 4·7); that is, they showed a secondary immune response.

The third experiment compared the immunological competence of lymph-node-originating clones with that of intrasplenic bone-marrow-originating noduli. A further control was added to test whether the antibodies measured in the previous experiments which had been formed following transfer of cloned cell populations were, in fact, produced by the cloned cells, or whether other cells of the lymph-node inoculum which could have migrated into the "clones" had participated in the immune response.

The following experiment was carried out: 70, female $(C3H \times C57BL)F_1$ mice were irradiated and inoculated, one group of 29 animals with 4×10^7 lymph node cells (donor group A), and a second group of 30 animals with 10^5 bone-marrow cells (donor group B), to produce bone-marrow clones. A third group (donor group C) served as an irradiated control. On day 8, all groups were immunized with shigella antigen and 3 days later the animals were killed and the spleen excised. A further 70, female $(C3H \times C57BL)F_1$ animals were now irradiated and divided into 5 groups. *Group* 1: 14 animals were each injected with the cell population of a single lymphoid clone from donor group A. *Group* 2: 26 animals were each injected with the cell population of a single bone-marrow-originating clone from donor group B. *Group* 3: 10 animals were injected with spleen cells from animals of donor group C. *Group* 4: 10 animals were injected with spleen cells from animals of donor group A which

TABLE III

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS

Group number	Treatment: X-irradiated mice inoculated with:	Number of reacting animals	Number of surviving animals	Per cent animals reacting	Titre log_2 produced by animals — Animals' numbers: 1	2	3	4	5	6	7	Mean titre log_2
1	Immunized, lymphoid clones	7	7	100	5	4	4	6	5	4	5	4·7
1A	Immunized, lymphoid clones; second immunization	5	5	100	10	8	8	4	8			7·6
2	Immunized, bone-marrow clones	0	10	0								0
2A	Immunized, bone-marrow clones; second immunization	0	12	0								0
3	8×10^6 spleen cells†; shigella antigen	0	8	0								0
4	8×10^6 spleen cells from donor group A; shigella antigen	0	10	0								0
5	Shigella antigen	0	4	0								0

† From immunized, X-irradiated donors.
For full explanation, see text.

did not show the formation of spleen nodules. *Group* 5: 10 animals were X-irradiated without cell inoculation. Two days later, 7 animals of group 1 (1A), 16 of group 2 (2A), and all animals of group 5 were immunized with shigella, and tested for antibody 6 days following immunization. The results (Table III) show that only animals inoculated with clones originated from lymph node cells formed agglutinin to shigella antigen. No antibody could be detected in animals inoculated with bone-marrow-originated clones. This is in accordance with our earlier demonstration of the immunological incompetence of cloned cell populations produced by inoculating bone-marrow or spleen cells into X-irradiated mice (Mekori and Feldman, 1966).

In the next series of experiments, we tested the immunological competence of lymph-node-originated colonies as measured by the graft-versus-host response. Parental-strain (female, C57BL) nodules were tested for their capacity to elicit splenomegaly in newborn $(C3H \times C57BL)F_1$ mice by determining the spleen indices according to Simonsen *et al.*, 1958. This splenomegalic effect was compared with that of parental, bone-marrow clones on newborn F_1 hybrid mice of the same strain. Irradiated C57BL mice were injected, 50 animals with 5×10^7 lymph-node cells from donors pretreated with PHA and 20 animals with 3×10^5 bone-marrow cells. On day 11, the spleen colonies were dissected out and cell suspensions from 2–3 individual clones were pooled, and injected into animals from two litters. *Litter* 1: five newborn animals were injected with 5×10^6 cells of lymph-node-originated clones and three served as non-injected controls. *Litter* 2: four animals were injected with 5×10^6 cells of bone-marrow clones and two served as non-injected controls. On day 11 following cell inoculation, the animals were killed and the spleen indices determined. The results (Table IV) show that cell populations of lymph-node-originated clones did elicit splenomegaly in newborn F_1 hybrids, whereas bone-marrow-originated clones did not. Thus, cloned populations derived from lymph node

cells are capable of manifesting a graft-versus-host immune response.

To test whether only cells from antibody-forming organs (that is, lymph nodes) are capable of giving immunologically competent clones, or whether thymus cells, when "cloned",

TABLE IV

INDICES OF GRAFT REACTION, C57BL → (C3H × C57BL)F_1, PRODUCED BY CLONED LYMPH-NODE AND BONE-MARROW CELLS

Control experiment	*Body weight* (*g.*)	*Spleen weight* (*g.*/100 *g.*)
Normal control	4·40	0·380
	4·50	0·270
	4·45	0·320
	5·20	0·390
	5·80	0·470
Mean of Control	4·87	0·366

Test experiment	*Body weight* (*g.*)	*Index*	*Spleen weight* (*g.*/100 *g.*)	*Index*	*Mean of indices*
Bone-marrow clones	4·80	0·985	0·350	0·956	1·005
	4·90	1·006	0·420	1·114	
	5·10	1·047	0·370	1·010	
	4·60	0·944	0·340	0·928	
Lymphoid clones	5·00	1·026	0·960	2·622	2·800
	4·80	0·985	1·050	2·868	
	5·10	1·047	0·980	2·677	
	4·90	1·006	1·050	2·868	
	5·20	1·067	1·100	3·005	

For explanation, see text and Simonsen *et al.* (1958).

will similarly manifest immunological reactivity, experiments were carried out in our laboratory by Alfah Peled. Cell suspensions of thymuses of 4-week-old C57BL mice were injected at doses of 5×10^7 cells into syngeneic recipients exposed to 900 r. (without pre-treatment with PHA). On day 11, the spleen colonies were excised and cell suspensions from pooled individual clones were

injected at doses of 5×10^6 cells into newborn (BALB/c × C57BL)F_1 mice to test for a graft-versus-host response. The results showed that cells of thymus-originating colonies elicited spleen indices of 2·55. To test if the immunological competence of thymus-originated colonies was achieved during their growth within the spleens of the primary recipients, a control group of newborn (BALB/c × C57BL)F_1 mice were each injected with 5×10^6 thymus cells from 4-week-old, donor, C57BL mice. The spleen indices obtained (2·88) were essentially similar to those produced by the cloned thymus cells.

DISCUSSION

The experiments reported in the present study have indicated that every clone of lymph node origin produced antibody to shigella antigen and haemolysins to sheep red blood cells, and that every population of 2–3 clones of parental-strain origin was found to give a positive graft-versus-host response when tested in newborn, hybrid F_1 mice. The fact that every clone formed antibodies to shigella antigen and haemolysins to sheep red cells is not attributed to selection of predetermined clones since, when the antigen was injected on the eighth day following cell inoculation, all clones were already well developed. Furthermore, the graft-versus-host reaction excludes this possibility since here the immunological competence was tested after the excision of the clones. Neither could the immune response be attributed to injected lymphocytes which had "contaminated" the spleen noduli. This explanation is improbable because spleen cells of animals which were treated with lymph node cells but failed to show any spleen colonies did not produce antibody to shigella antigen. We therefore feel justified in suggesting that every clone can form antibody to any of the antigens tested; that is, that every clone is, in fact, pluripotential in terms of antibody production.

This obviously need not mean that *every* cell within a cloned population is pluripotential. Heterogeneity could have evolved within a cloned cell population if the genes coding for γ-globulin chains have a high mutation rate, as suggested by Burnet (1959, 1964), and if mutational changes involving such genes have taken place during the exponential replication of the clone-forming cells. However, even if one assumes a high mutation rate of 10^{-4} (Burnet, 1964) and a modest estimate of 5×10^5 antibodies, one would not expect the clones to manifest a uniform reactivity to three randomly chosen antigens. A population of $(1-2) \times 10^6$ cells would not accumulate a uniform distribution of such mutants. Our results do not seem reconcilable with the notion that each immunologically competent cell is genetically predetermined to form one specific antibody, but rather that immunologically competent cells are *a priori* pluripotential. Whether the state of pluripotentiality is based on the pre-existence in every cell of all cistrons required to form the whole spectrum of antibodies, or whether it is based on an "instruction" type of mechanism remains a subject for further studies.

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DISCUSSION

Burnet: The evidence that these nodules are clones is really that they have been initiated by single cells. The weakness in the discussion, as Dr. Feldman has pointed out, lies in the paucity of evidence that they are clones in the sense that all their components are descendants of individual cells. Professor Gowans and I have discussed before what seems to me to be a very important point: the possibility that, in any intact animal or for that matter in any functioning animal, the lymphoid cell population of any accumulation of lymphoid cells in the body is very nearly a cross-section of the average lymphoid cell population of the whole body. It will always be extraordinarily difficult to

devise a critical experiment to test a clonal concept of this sort in a whole mammalian organism. We shall always be defeated by the mobility of lymphoid cells in the circulation and within the tissues. This objection has to be countered before you can persuade me, or I think the majority of immunologists, that every cell is immunologically totipotent.

Nossal: Dr. Feldman has told us that there are no very good analogies for restrictions in the potentialities of lymphoid cells other than those based on mutations, but there are, of course, Sonneborn's experiments with paramecium and its flagellar antigenic variations (Sonneborn, T.M. [1947]. *Adv. Genet.*, **1**, 264). Even if we accept *in toto* the evidence that Dr. Feldman has presented this by no means deals a death blow to the theory of clonal selection. The most important aspect of clonal selection is what is the potentiality of any particular immunologically competent cell at any particular moment in time. As long as we accept heterogeneity, the central facets of both the immune response and of tolerance can be explained. In other words, if in a normal animal at a given moment in time this fellow is making hay and that fellow is asleep I do not think that the fact that the sleeping fellow may be potentially able to make hay really matters very much. Why could we not have a situation in which, in antibody-forming cells, a particular gene on a particular chromosome is active, as in a bacterium which is fermenting lactose? Recognition could be mediated by antibody on the surface of the cell which confers reactivity to a particular antigen. When phytohaemagglutinin is introduced, a central control characteristic may be blotted out and a phenotypically restricted cell may be turned into a pluripotential cell. After all, it is only the descendant of a fertilized ovum.

Feldman: I agree with you. The experiments I have described do not eliminate the possibility that some type of heterogeneity may characterize the lymphoid-cell populations of an organism. Our experiments do, however, seem to be incompatible with the notion that *mutational* processes involving genes which determine the γ-globulin chains are the basis of this heterogeneity. Our work suggests that the immunologically competent cells have the same pattern of genetic information for antibody production as other cells and this, as you know, was not the original idea of the clonal selection concept of antibody formation.

Metcalf: H. Friedman (1965. *Proc. Soc. exp. Biol. Med.*, **118**, 1176) recently reported that neonatal thymectomy reduces both the number of unprimed, plaque-forming cells and the rate at which these cells proliferate after the administration of sheep red blood cells. Dr. Feldman, have you ever tried to put cells into a thymectomized recipient animal and, if so, has this affected the proliferation in the spleen colonies of the plaque-forming cells?

Feldman: We have thought about this but we have not actually done it.

Good: The last part of your presentation, Dr. Feldman, is a very interesting demonstration of a concept we have been following up that was first proposed by Warner and Szenberg (Szenberg, A., and Warner, N. L. [1962]. *Nature, Lond.*, **194**, 146). This suggests that the thymus and the thymus-dependent system of cells, in their ultimate differentiation, may be very restricted in what types of immune responses they can exercise and that these do not necessarily involve synthesis of the kind of antibodies that get into the circulation. It might be interesting to mix cells from heavily irradiated donors with the thymus population that you believe you are cloning out. In this way one might introduce another population of cells into your system and any of the cells which were radioresistant would represent, in the mammal, cells with a different origin from the thymus cells. With this method a mixture of cells of the immunoglobulin system would result and these could then be tested for antibody production. What I am driving at is that in your antibody-producing clones derived from the peripheral lymphoid tissue, there appear to be two kinds of cells; in the clones derived from the thymus, only one. Therefore, the cells of the pure thymus system should, as you found, give rise to cellular immunity only, whereas the cells from the spleen which contains the two populations, could give rise to both cellular immunity and free antibody. Is it possible, Dr. Feldman, that the cells in your experiments home together to produce what I would call a mixed clone? Have you tried the sort of study I have just described?

Feldman: No, we have not done this. We have recently been looking for a system for priming macrophages. This is associated with our studies on the induction of tolerance following sublethal doses of irradiation (Nachtigal, D., and Feldman, M. [1963]. *Immunology*, **6**, 356;

[1964]. *Immunology*, **7**, 616). We aimed at testing whether tolerance is determined by the direct interaction of the antigen with lymphoid cells (in the absence of macrophages), in contrast to the induction of antibody which may require the processing of the antigen by macrophages. With Ruth Gallily of our laboratory, we have injected into sublethally irradiated mice, macrophages which had interacted *in vitro* with shigella antigen. These mice do not respond to shigella antigen when it is injected after total-body irradiation. However, our preliminary results indicated that the mice did produce antibody when injected with macrophages which had interacted with shigella antigen. Experiments on the minimal number of macrophages which are needed to initiate an immune response will indicate whether any heterogeneity exists within the macrophages, associated with the heterogeneity of antigens.

Rowley: If we accept the probability that both macrophages and lymphocytes are necessary for antibody production, that lymphocytes may be pluripotential and that macrophages, in order to take up antigenic material, must contain antibody against that material, then we have to consider a macrophage-clonal selection theory in which the determinant for selection is the ability of the macrophage to take up antigen. This is not an original suggestion of mine.

Šterzl: After we had read Dr. Feldman's recent paper in *Nature* (Mekori, T., Chieco-Bianchi, L., and Feldman, M. [1965]. *Nature, Lond.*, **206**, 367) we were able to reproduce your model. Different doses—2×10^7, 10^7, and 5×10^5—of the lymphoid cells isolated from a phytohaemagglutinin-stimulated donor were injected into lethally irradiated syngeneic recipients. Nine days after cell transfer, the mice of different groups were injected intravenously with 0·5 ml. of a 1 per cent suspension of sheep erythrocytes. Four days later, cells from individual clones of the spleens of irradiated recipients were isolated and the number of antibody-forming cells estimated by the plaque technique. As in normal mice (Šterzl, J., and Mandel, L. [1964]. *Folia microbiol., Praha*, **9**, 173) around 20,000–40,000 antibody-forming cells were detected per 10^8 lymphoid cells. If we accept that four days after immunization the majority of antibody-producing cells arise through mitosis, then only a relatively small number of cells reacted with the appropriate antigen at the beginning of our experiments, probably not more than 100–200 cells per 10^8 lymphoid cells. Dr. Feldman, how do

you explain why only such a small number of cells having, according to your conclusions, multipotential capacity responded to the injected antigen? It is difficult to explain this situation if we accept that the very low number of immunologically competent cells (for sheep red blood cells) in the clone is genetically determined. I do not see how these individual cell differences can occur when the clone develops from only one (or a few) cells. It may be assumed that, during clone formation, either a process of differentiation and repression in individual cells takes place or that the cells' different potentialities result from random mutations. The only other possible explanation that occurs to me is that in the lethally irradiated mice not all immunologically activated cells were killed. The surviving cells (of recipient origin) had already been activated by previous contact with antigenic determinants common to sheep red cells, and migrated into the clone produced by the donor cells.

Feldman: The reason that only a small proportion of the lymphoid-cell population responds to an antigen at any given time is obviously very important, and as yet unknown. The fact that the distribution of plaque-forming cells in the cloned population is similar to their distribution in the normal cell population is another indication that mutational processes cannot explain the heterogeneity of the lymphoid system.

Your final comment, Dr. Šterzl, concerning cell contamination, is a very serious one although the reasons for my anxiety about it are different from yours. Contamination of the cloned cell populations is expected to occur not through cells of recipient origin but rather through donor cells that do not participate in colony formation. In our studies with shigella antigen we found that cells of the interclonal splenic areas did not react to the antigen. But in our experiments with sheep red blood cells, cells of interclonal origin did produce haemolysins and, according to our controls, these cells were of donor origin. The possibility of contamination of the cloned population by non-cloned cells in this system is now being studied.

Auerbach: If we look at your data from a slightly different view point, is it not possible that in the population of injected cells there are a few embryonic-type cells? In other words, we know that in animals there is a very gradual loss of tolerance of some cells with age

and that it is these cells that may be clonable. Therefore the spectrum of cells that is present in your systems should be similar to a complete embryo's cells and have the same mechanism for differentiation as do the cells of an embryo. This would not be because of any sort of counter-selection but simply because you originally selected that low number of cells which had never gone through the process of differentiation.

Feldman: I agree, but since the cell population that we measure within a colony is of a limited size the events leading to manifestations of heterogeneity within a colony cannot be mutational.

THE THYMUS IN RELATION TO IMMUNOLOGICAL TOLERANCE

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Antigen can drive lymphoid cells in two basic directions: towards immunity or towards tolerance. We know much about some of the factors, such as antigen dose and age of the animal, which influence the direction taken, but the detailed cellular events involved are still obscure. Recent studies in our laboratory have been directed at finding out more about how antigen works. We have followed the distribution of ^{125}I-labelled antigens in adult non-immunized (Nossal, Ada and Austin, 1964), immunized (Nossal *et al.*, 1965) and tolerant (Ada, Nossal and Pye, 1965) rats. In this review, we wish to present some newer findings on the distribution of antigen injected into newborn rats. Such antigen induces immunological tolerance, and the observed differences in localization pattern from that seen in adults may aid our understanding of the mechanisms of action of antigen.

THE BLOOD-THYMUS BARRIER FOR ANTIGENS

The concept of a blood-thymus barrier for antigens in adult animals is well established, though all are agreed that the barrier is not a complete one (Kaplan, Coons and Deane, 1950; Marshall and White, 1961; Sainte-Marie, 1963; Weiss, 1963; Clark, 1964). We were interested to compare the effectiveness of this barrier (*a*) in adult and newborn rats and (*b*) for various types of antigens. For descriptive purposes it is convenient to separate the problem

into two phases: initial penetration and subsequent persistence of antigen. Some data on initial penetration are presented in Table I and Fig. 1.

Bovine serum albumen (BSA) and three flagellar antigens from *Salmonella adelaide* (flagella, polymerized flagellin and flagellin) were used. BSA has a molecular weight of approximately 60,000; flagella and polymerized flagellin represent particulate antigens containing hundreds of flagellin subunits per particle; and flagellin is a soluble protein with a molecular weight of approximately

TABLE I

RATIOS OF SPECIFIC ACTIVITIES (COUNTS/SEC./MG. WET WEIGHT) OF VARIOUS ORGANS TWO HOURS AFTER THE INJECTION OF VARIOUS LABELLED ANTIGENS INTO ADULT AND NEWBORN RATS

Antigen	*Adult*	*Newborn*
Flagella	19† : 1·5 : 1	2·9 : 2·4 : 1
Polymerized flagellin	10 : 4 : 1	1·3 : 3·1 : 1
Flagellin	2·6 : 11 : 1	1 : 1·9 : 1
BSA	1·2 : 2 : 1	0·5 : 0·7 : 1

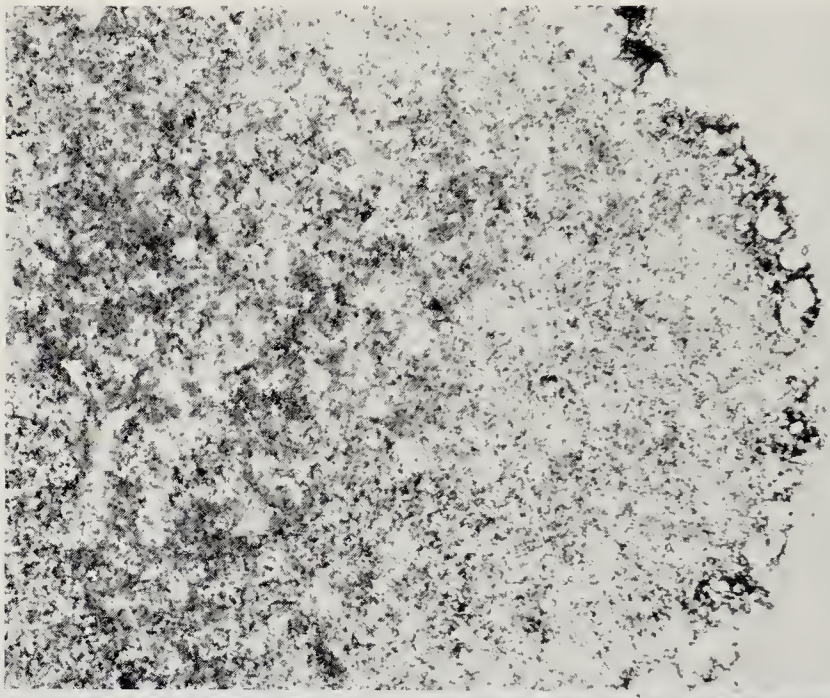
† In each case the first value represents spleen, the second mesenteric lymph node and the third thymus.

38,000. These antigens were iodinated by oxidation with chloramine T (Greenwood, Hunter and Glover, 1963) and doses of 10 to 100 μg. (0·2 to 2 mc) were injected, intravenously into adult and intraperitoneally into newborn rats. Table I shows the specific activity (counts/sec./mg. tissue) ratios of spleen:mesenteric lymph node:thymus, two hours after injection of antigen. In adult animals, the particulate antigens flagella and polymerized flagellin were well trapped in the spleen, the former more so than the latter. The spleen:thymus ratios were high, but the lymph node:thymus ones lower. The soluble antigens with lower molecular weights, flagellin and BSA, were poorly retained by the spleen; in fact, higher specific activities were achieved in lymph nodes than in spleen. It is of interest to note that BSA has a higher molecular weight than flagellin and yet the lymph node:

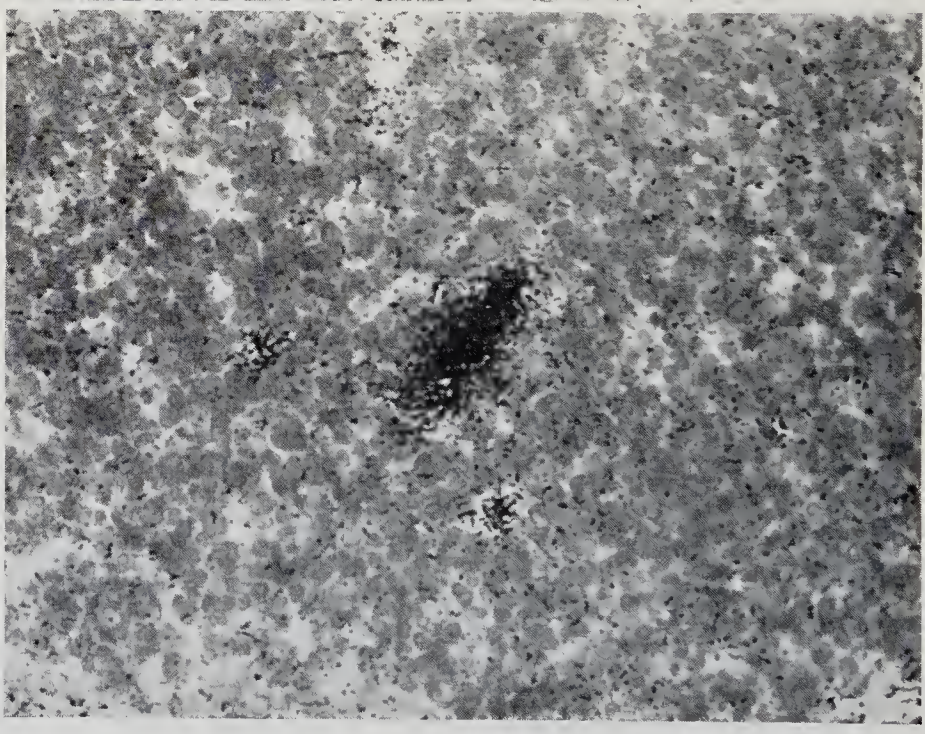
thymus ratio was much higher for flagellin than for BSA, suggesting that the effectiveness of the "barrier" may depend on factors other than molecular size. In newborn animals the distribution of radioactivity was much wider. Even with the particulate antigens the spleen was only somewhat more radioactive than the thymus, and with BSA the thymus actually contained more isotope than the other lymphoid organs. Thus the counts on the whole support the conclusion that the barrier is ineffective in newborn rats.

The specific activity data alone are deceptive in a number of ways. First, the blood content of the three organs concerned varies greatly. Second, no discrimination is made between isotope in phagocytic cells and isotope diffusely distributed through the lymphoid tissue. Third, we have noted that the thymic capsule can be very heavily labelled and can thus contribute disproportionately to the overall picture. Thus, detailed information on a blood:thymus barrier must be obtained from quantitative radioautography.

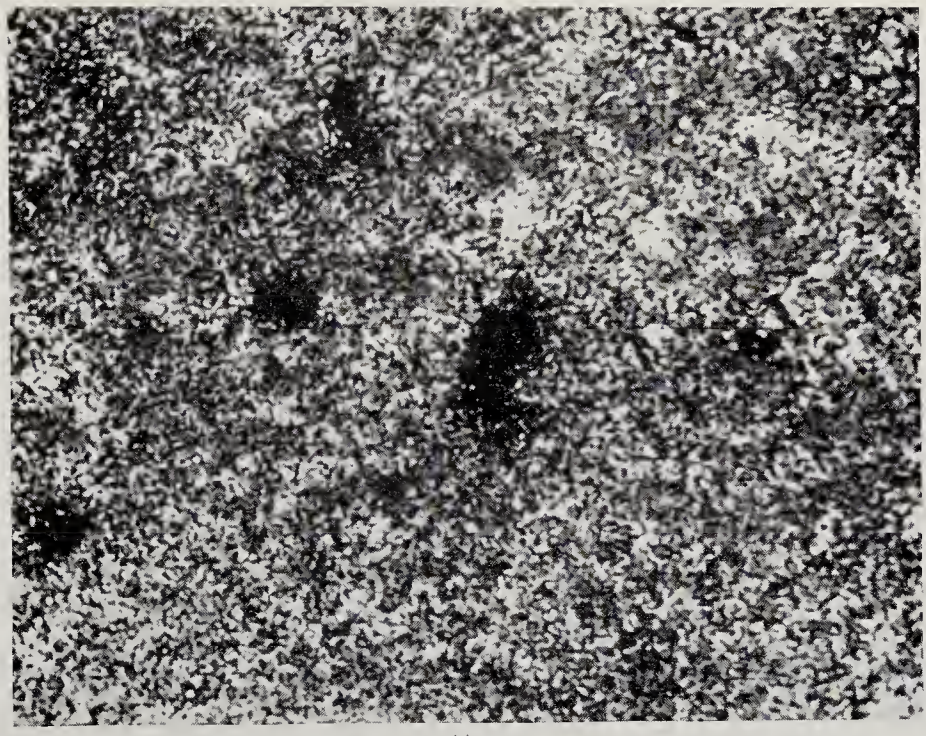
Fig. 1 shows labelling patterns that were typical of much of this work. The popliteal lymph node and thymus of an adult animal and the thymus of a newborn rat are compared two hours after injection of ^{125}I-labelled BSA. The adult lymph node shows diffuse permeation of label throughout the cortex. The adult thymus shows a blood vessel whose lumen is heavily labelled; there is also some slight but definite labelling over the lymphoid cells of the surrounding cortex, but this is much lighter than in the lymph node. In the newborn thymus the blood vessels are also outlined by confluent label, but there is much wider dissemination of antigen throughout both cortex and medulla. These and many similar radioautographs confirmed that there is a partial blood–thymus barrier in adult animals but that this is much less effective in the newborn. This held true even for the particulate antigens. With flagella and polymerized flagellin in the adult, little label escapes into the thymus cortex



(a)



(b)



(*c*).

FIG. 1. Radioautographs of sections from rats 2 hours after injection of ^{125}I-labelled BSA. (× 250). (*a*) Cortex of popliteal lymph node of an adult rat. Note diffuse labelling. (*b*) Cortex of thymus of an adult rat. Note heavily-labelled blood vessel and slight penetration of label into the lymphoid area. (*c*) Cortex of thymus of a newborn rat. Note confluent labelling over vessels, and extensive penetration of label into the cortex.

and most of the observed counts are attributable to antigen in vessel walls, perivascular phagocytes, capsular labelling and rare scattered phagocytes in the medulla. In the newborn, even the particulate antigens penetrate extensively into the thymus cortex and medulla.

PERSISTENCE OF ANTIGEN WITHIN THE THYMUS

In adult animals one striking feature which distinguishes the thymus from other lymphoid organs is the absence of a specific antigen-retaining mechanism. One day after antigen injection,

the phagocytic cells in the perivascular connective tissue are already much lighter and within two days thymus labelling is essentially down to background levels. In contrast, disappearance of antigen from the thymus in newborn rats occurs much more slowly. Again, there are few labelled phagocytic cells except

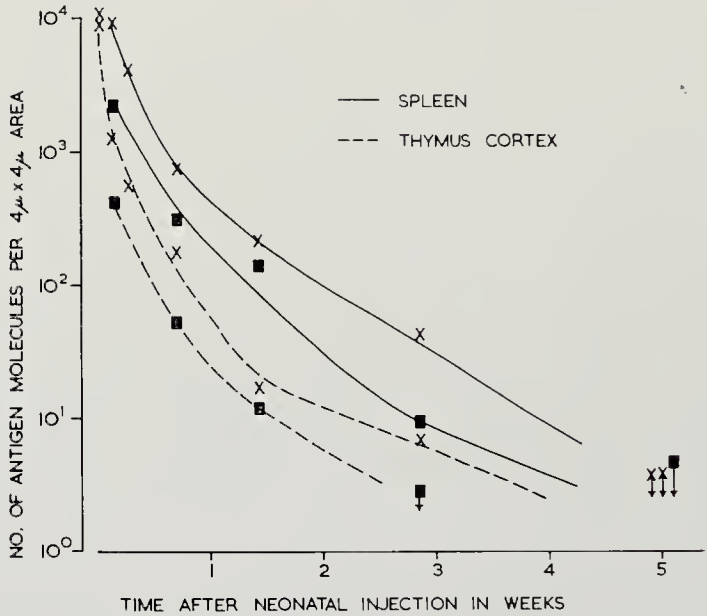


FIG. 2. Number of molecules of antigen persisting over lymphoid areas of spleen and thymus cortex after single injection of 100 μg. of ^{125}I-labelled salmonella flagellar antigens. Each 4μ × 4μ area covers approximately one small lymphocyte. ×——×, polymerized flagellin in spleen, ×----× in thymus. ■——■, flagellin in spleen, ■----■ in thymus.

those associated with vessel walls. Most of the label is diffusely distributed over the thymic cortex and medulla and disappears at a slow rate, presumably by progressive catabolic decay. This feature of persisting thymic labelling is illustrated in Fig. 2, where the number of molecules of antigen per cell area has been derived by a method previously described (Nossal, Ada and Austin, 1965*a*). After a single pulse of antigen given on the day of birth, label persists over the thymic cortex for three weeks or longer.

COMPARISON OF ADULT AND NEWBORN LABELLING PATTERNS IN LYMPHOID ORGANS OTHER THAN THE THYMUS

We have described elsewhere the distribution of antigens in the lymph nodes (Nossal, Ada and Austin, 1964) and spleen (Nossal, *et al.*, 1966) of adult animals. Antigen localizes in two types of cells: the web-like dendritic macrophages of lymphoid follicles; and the less specialized reticular cells of the lymph node medulla and spleen red pulp. For the flagellar antigens the trapping mechanism is effective and, within a day, virtually all the antigen has either reached phagocytic cells or has been catabolized and excreted. The picture in the newborn is very different. Newborn rats possess phagocytic cells, as shown by studies on the uptake of colloidal carbon or whole bacteria (Suzuki, 1957; Reade, 1964). However, phagocytosis of the smaller foreign entities (both soluble proteins and flagellar particles) used in our studies was surprisingly inefficient during the first few days. Some phagocytosis definitely occurred, but the majority of the antigenic material in all lymphoid tissues lay scattered diffusely over the whole section. Resolution was not good enough to allow us to determine whether the antigen was in, on, or between the lymphocytes. Fig. 3 shows the weak phagocytosis of flagellin in newborn as opposed to adult rat spleen, even four days after injection. In the adult, the antigen was in the characteristic semilunar web on the outer aspect of the germinal centre. In the newborn, scattered labelled cells and much unphagocytosed antigen were evident. This diffusely spread label persisted for four weeks or longer (see Fig. 2). All the lymphoid organs showed this pattern of persistence of non-phagocytosed material with all four antigens.

Interestingly, a pattern not greatly different from the distribution of flagellar antigens in newborns was found following the intravenous injection of BSA into *adult* rats. Phagocytosis was minimal and wide distribution evident. However, radioauto-

graphs, as in Fig. 1(*b*), showed a partial blockage of entry into the thymic parenchyma. The wide cellular dissemination of BSA

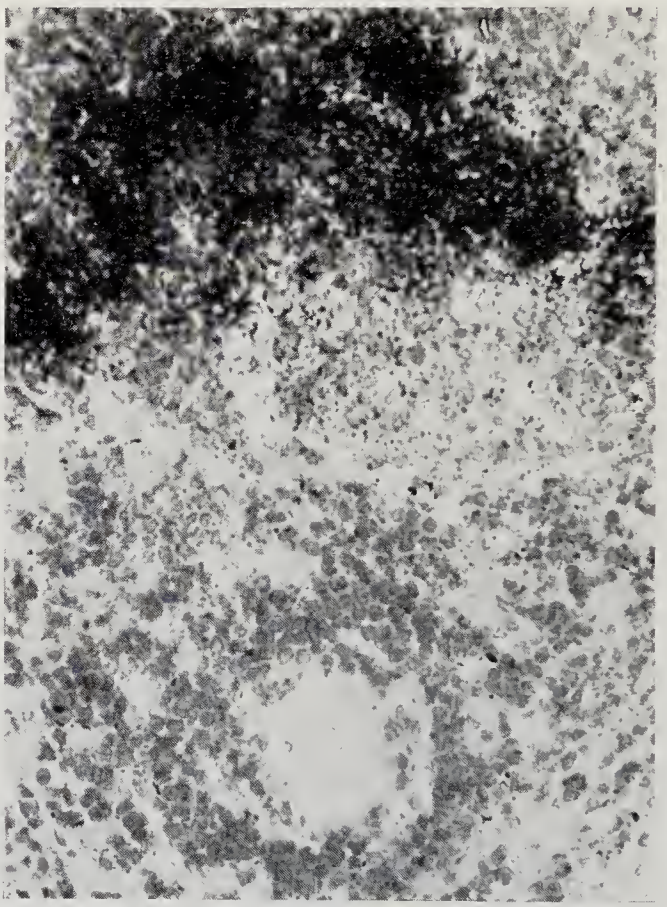


FIG. 3(*a*)

FIG. 3. Radioautographs of spleen sections from rats given ^{125}I-labelled flagellin 4 days previously. (× 250).

(*a*) Adult rat. Note typical semilunar cap of label on outer aspect of germinal centre. The rest of the white pulp (around the central arteriole at the bottom of picture) is unlabelled.

in adults was of special interest to us as this is an antigen with which adult tolerance can be induced fairly readily (Mitchison, 1964).

OTHER ANTIGEN-PROTECTED SITES

The thymic cortex is by no means the only portion of the lymphoid system of adults to which antigens have limited access.

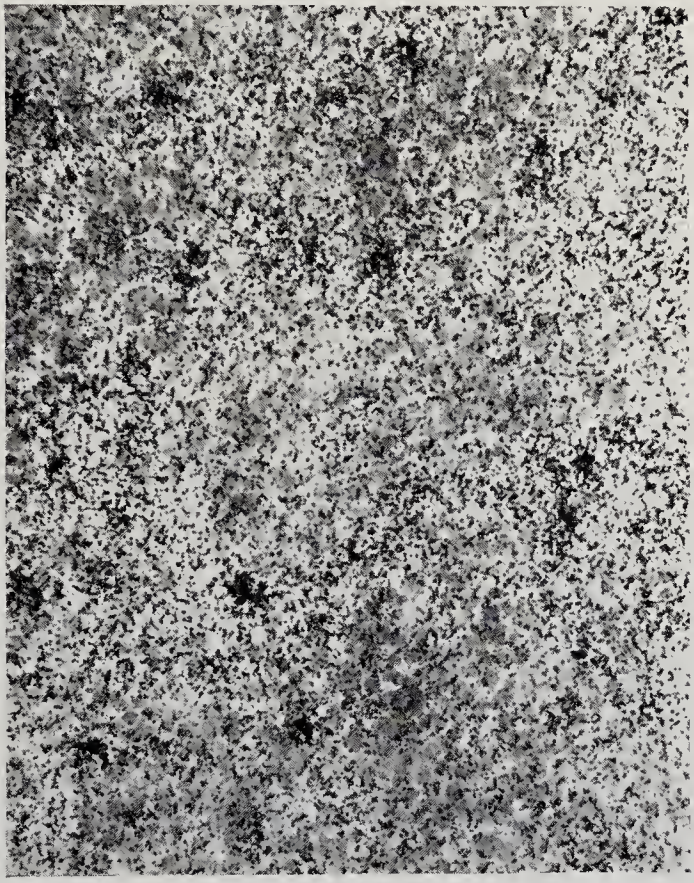


FIG. 3(*b*)
(*b*) Newborn rat. Note ineffective phagocytosis and diffuse distribution of antigen.

This was especially true for the particulate antigens. During the first few hours after injection of these materials, the white pulp of the spleen and the cortex of the lymph nodes contained much less antigen than, respectively, the red pulp and the lymph node medulla. The follicular areas in particular were spared the flooding with large amounts of free antigen. Within about four

hours, the dendritic network of the follicles began to show label; the small amount of antigen which did penetrate to the diffuse lymphoid tissue of splenic white pulp or lymph node cortex rapidly disappeared. In fact, when grain counts were performed over these areas in adult animals, just as good a case could be made out for a "blood-diffuse cortex" barrier as for a "blood-thymus" barrier. This was less true for the low-molecular-weight flagellin and, as mentioned above, was not true for BSA.

LOCALIZATION OF ANTIGEN IN RATS GIVEN MULTIPLE INJECTIONS OF ANTIGEN FROM BIRTH

Single injections of flagellar antigens given on the day of birth can produce immunological tolerance, but this is transient and frequently only partial (Nossal, Ada and Austin, 1965*b*). More complete and lasting tolerance can only be produced by an injection course. We have attempted to trace the fate of ^{125}I-labelled flagellin molecules injected into rats twice-weekly from birth onwards. The dose used was 1 μg. per injection, the minimal dose effective in tolerance induction. The substitution rate was kept as close as practicable to 1 atom of ^{125}I per molecule of flagellin. By exposing radioautographs for 60 days, one could, in this way, trace amounts of antigen as small as 5–10 molecules (Nossal, Ada and Austin, 1965*a*). Of course it was not possible to know if a given silver grain reflected antigen given in the first, second, third or subsequent injections. However, a general picture did emerge, and the data are relevant to the antigen content of "tolerant cells". During the first week of the course of injections the diffuse labelling pattern already described was observed. By one week, phagocytosis was beginning but large amounts of antigen persisted diffusely. Labelled phagocytes tended to spare the developing splenic white pulp. By two weeks, penetration into the thymus was beginning to diminish and the relative

sparing of the splenic white pulp was more obvious. However, phagocytosis was still relatively inefficient, follicular localization was not evident, and diffuse labelling was quite heavy. By four weeks, the "blood–thymus barrier" appeared to be relatively effective. The spleen at this time-point showed interesting features: the white pulp contained no germinal centres but rounded collections of lymphocytes representing primary follicles were seen. These showed excellent and characteristic labelling. None the less some diffuse label persisted. By six weeks, the labelling pattern approached that seen in adult animals. Thymic labelling was scarcely above background, follicular labelling was noticed in the spleen and lymph nodes and phagocytosis had occurred in spleen red pulp and lymph node medulla. After this time-point, diffusely scattered labelling occurred only in animals killed within a few hours of antigen injections.

In summary, our radioautographic data showed the presence of diffuse labelling of diminishing degree over the first four weeks, accompanied by the progressive development of an adult-type localization pattern. By eight weeks, the animals, though fully tolerant, handled the newly injected antigen in an unmodified way.

THE ANTIGEN CONTENT OF LYMPHOID CELLS IN ADULT, TOLERANT ANIMALS

Previous work has shown that mature antibody-forming plasma cells contain no detectable macromolecular antigen (Nossal, Ada and Austin, 1965*a*). Apparently antigen need not persist in a cell for continued antibody synthesis. The present experiments indicate that antigen does not have to persist in lymphoid cells to maintain tolerance. This is evident in the data presented in Fig. 2. Let us presume that most of the scattered label over, for example, the spleen section represented antigen inside lymphocytes. The minimum dose of monomeric flagellin

capable of inducing tolerance when given as a single injection to newborn rats is 100 μg. Animals given such a single pulse are tolerant, though not always completely tolerant, at ten weeks of age. Yet the level of antigen per small lymphocyte in the splenic white pulp fell to 10 molecules per cell at three weeks and, by extrapolation, to 10^{-3} to 10^{-4} molecules per cell at ten weeks. In the experiments in which multiple doses of 1 μg. of ^{125}I-labelled flagellin were used to induce tolerance, the experimental design involved a series of iodinations, and some variation in substitution rate could not be avoided. This made calculation of exact antigen levels difficult. However, inspection of sections of adult, fully tolerant animals showed that the lymphoid cells contained no detectable antigen following the cessation of the injection course. The only cells showing labelling above background were specific antigen-capturing cells including follicular reticular cells. We realize de-iodination and/or fragmentation may occur and that radioactively labelled material may be lost during fixation and histological processing. However, subject to these limitations, it appears that the lymphoid cells in adult tolerant animals contain less than, and possibly much less than, one molecule of antigen.

THEORETICAL CONSIDERATIONS

How are these findings relevant to the problem of the induction of immunity or tolerance? The predominant impression emerging from this work is that in circumstances where *immunity* is the final outcome of the injection, phagocytosis and specific retention of antigen are dominant features; whereas in circumstances favouring *tolerance* induction, diffuse dissemination of the antigen round the whole lymphoid system, including the thymus, is observed. Such a correlation, of course, proves nothing. However, it is consistent with the view first expressed by Medawar (1963) that the usual result of an encounter between native antigenic molecules and immunologically competent cells is

tolerance induction; whereas antigen presented "via the proper diplomatic channels", that is after due processing by macrophages, leads the cell to differentiate towards immunity.

At first sight the notion that cells can be made tolerant so readily is difficult to accept. However, it must be stressed that in any natural immunological situation the antigen (bacterium or virus) is a particulate entity. Furthermore, natural opsonic factors appear to exist for such infectious agents (Rowley and Jenkin, 1962). Thus the number of lymphoid cells which would meet up with free antigenic determinants would be very small, phagocytosis would be rapid, and most lymphocytes would only encounter antigen in the course of their wanderings through the lymphatic tissues. The cells would enter lymph nodes via the post-capillary venules and would sooner or later move to the follicular area. Here they might be "caught" in the dendritic web of antigen-retaining cytoplasmic processes (Miller and Nossal, 1964). In this intimate relationship between reticular cell and lymphocyte the initial events of immunity induction (whatever these may be) might occur. On the other hand, "self" antigenic determinants, present at all times in high concentrations in the circulation, would frequently meet with lymphocytes. As such "self" proteins are not well phagocytosed, particularly in the lymph node cortex (Ada, Nossal and Austin, 1964), the chances of encounter between lymphocytes and antigen-retaining macrophages are much smaller. Thus tolerance is the final outcome.

The important experiments of Mitchison (1964) seem to us to give support to these ideas. Mitchison, using BSA in adult mice, has shown conclusively that tolerance and immunity can be simultaneously induced in an animal. Thus, some members of the lymphoid population are recruited into antibody formation and others into tolerance. It is of particular interest to us that he was using BSA, an antigen which we have shown to be widely disseminated through the lymphoid system after intravenous

injection into adult animals. It seems to us that the common link between the "low-dose paralysis" induced by Mitchison in adult mice with BSA, and the tolerance which we have induced with microgram doses of flagellin in newborn rats, is ineffective phagocytosis. In Mitchison's experiments, the antigen is not palatable to the phagocytes, possibly because of a low level of opsonins. In our work, the phagocytic system is not yet functionally mature. In this connexion, it is particularly interesting to note that Mitchison failed completely to induce tolerance with alum-precipitated BSA which, presumably, would have been much more extensively phagocytosed.

If this overall picture is correct, it is important to note the small doses of antigen which suffice to cause paralysis. In both the BSA and flagellin systems, around 10^{13} molecules (injected repeatedly) suffice. In our multiple-dose experiment, the level of antigen observed in lymphatic tissues just before an antigen pulse was not much greater than one molecule per lymphocyte. It appears that prolonged exposure of the lymphocyte population to a constant level of free antigen of over one molecule per cell for several weeks leads to tolerance. Once the tolerant state is achieved, the actual presence of antigen in lymphoid cells is no longer required. The animal can remain tolerant long after the antigen has either been catabolized or mopped up by phagocytes.

SPECULATIONS ON THE TOLERANCE MECHANISM

In speculating about possible mechanisms of tolerance induction at the cellular level, we must now take note of two new facts: (a) tolerance can be induced by low, persisting concentrations of antigen; (b) tolerance can persist though no antigen remains in the tolerant cell population. The simplest explanation for this seems to be provided by a "clonal deletion" theory of tolerance (Burnet, 1959). Let us postulate that small lymphocytes synthesize, and carry on their surface, small quantities of γM-"natural"

antibody; let us speculate further that each lymphocyte at any given time synthesizes only one particular type of antibody-combining site. When a lymphocyte encounters antigen capable of reacting with the surface γM-antibody, quite small amounts may suffice to fix complement and cause lysis and death of the lymphocyte. If this happens to *all* the cells capable of reacting with that antigen, the whole animal is rendered tolerant. If, on the other hand, the cell is "fed" antigen-RNA complexes or some other material from adjacent macrophages, the events leading to plasmacytopoiesis may occur.

THE ROLE OF THE THYMUS

The thymus may be important in tolerance mechanisms in at least two ways. First, some immunologically competent cells may later be seeded out to the lymph nodes and spleen (Nossal, 1964). In that case, antigen would have to reach these cells in the thymus cortex to ensure their "clonal deletion". The partial blood-thymus barrier may make this difficult. In some circumstances, for example with BSA in adult rats, the concentrations of antigen in the thymic cortex could be the limiting factor in tolerance induction. However, with most antigens, there are other antigen-protected sites apart from the thymus (for example, the diffuse cortical tissue of lymph nodes). The effects of adult thymectomy on the dose of antigen needed to induce tolerance might then vary from system to system.

Secondly, the thymus may be of great importance in deciding whether tolerance is maintained or broken down (Claman and McDonald, 1964). If all the lymphoid cells of a given "clone" are deleted at one time-point the rate of regeneration of that immunological potential might well depend on the rate of formation of new "virgin, immunologically competent cells". This process, which we have termed lymphoneogenesis (Nossal, 1965) may occur in the thymus, and certainly appears to require a

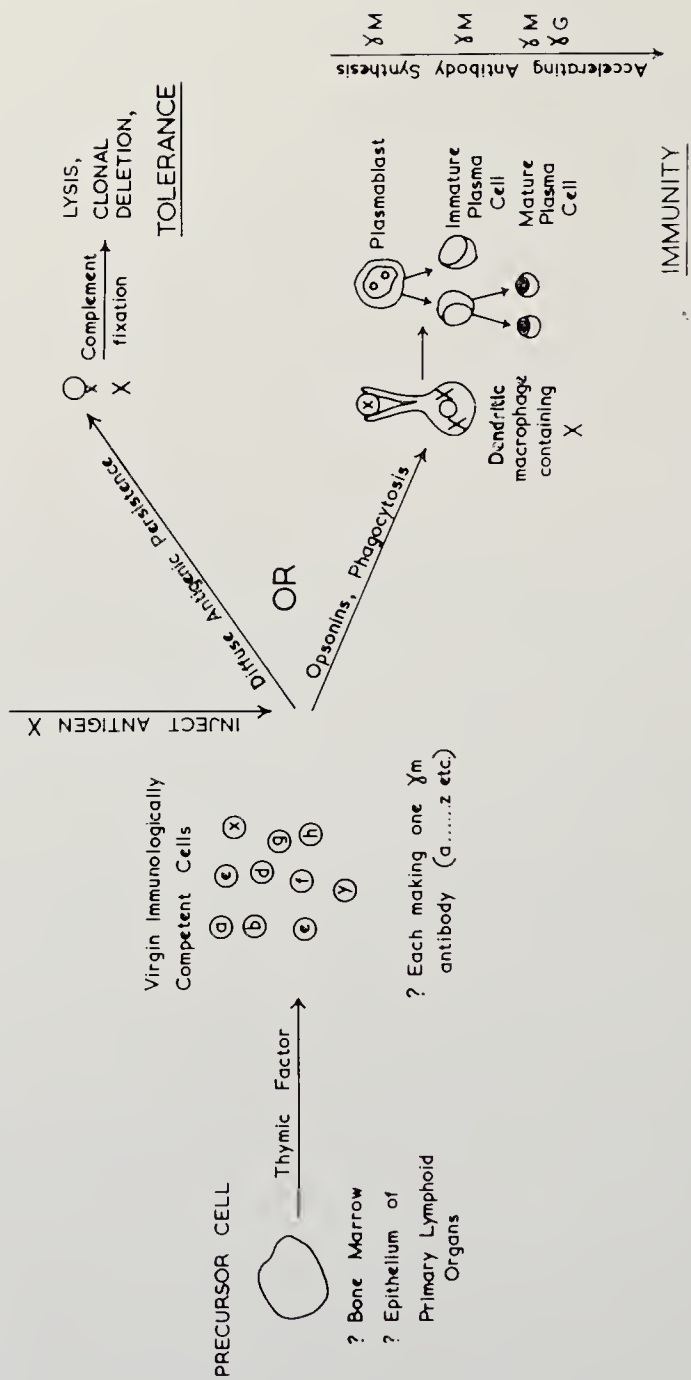


FIG. 4. Speculative mechanism for the induction of tolerance and immunity.

thymic factor (Harris and Ford, 1964; Osoba and Miller, 1963). Even if a single pulse of antigen has "deleted" all the cells of a given clone, or has in some other way rendered the population tolerant, immune reactivity may shortly reappear through differentiation of new lymphocytes under thymic influence. Permanent tolerance could then only be achieved by one of two means: (a) thymectomy (presuming there is no other organ which shares the thymic function); (*b*) maintenance of diffuse antigenic persistence until all lymphoneogenesis had ceased. This might be achieved by giving either a substantial dose of antigen at birth (Humphrey, 1964) or repeated antigen doses until full adult life (Nossal, Ada and Austin, 1965*b*).

This theoretical and speculative analysis is summarized in Fig. 4.

SUMMARY

(1) There is a partial blood–thymus barrier for antigens in adult animals. This cannot be regarded purely as an anatomical entity. Rather, it is a functional concept depending, amongst other things, on the rapidity of phagocytosis of antigens.

(2) The effectiveness of the blood–thymus barrier varies for different antigens. Particulate antigens enter less readily than soluble antigens. However, molecular size is not the sole determining factor as bovine serum albumen, though about twice as large as flagellin, penetrated more extensively than the latter into the thymus cortex.

(3) The blood–thymus barrier is much less evident in newborn than in adult animals. Whether this is due to an anatomical or a functional difference has not been established.

(4) When radioactively labelled antigens are injected into newborn and adult animals there are major differences in localization pattern. In the adult, retention, particularly in lymphoid follicles, is the key finding. In the newborn, diffuse spread and prolonged persistence of antigens outside phagocytes is the rule.

(5) Tolerance induction appears to be favoured by the continued presence of antigen on or in lymphocytes at or above a mean level of one molecule per cell.

(6) Once tolerance has been induced, it can be maintained for some time without the physical presence of antigen in the lymphoid cells.

(7) A speculative model of tolerance induction has been presented. It is an elaboration of Medawar's notion that direct contact of antigen with immunologically competent lymphocytes leads to tolerance, whereas stimulation depends on the combined activity of macrophages and lymphoid cells. It is proposed that lymphocytes of a specific clone carry some γM-antibody on their surface, and that when they encounter even a few molecules of antigen an antigen-antibody union occurs leading to cellular destruction, possibly due to complement action.

(8) Persisting tolerance depends on (a) antigen reaching all lymphoid areas including the thymus; (b) antigen persisting while lymphoneogenesis under thymic influence persists.

ACKNOWLEDGEMENTS

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DISCUSSION

Ito: I have studied the relationship of blood vessels to the thymic parenchyma. On electron microscopy, the thymic capillaries often seem to be surrounded by a continuous layer of epithelial cells and separate from the cortical parenchyma, that is, from the parenchymal lymphocytes within the epithelial framework. This appearance may suggest the presence of a blood-thymus barrier. But when cortical capillaries are traced under low magnification the perivascular space is

found in places to comunicate directly with thymic parenchyma (Fig. 1). In the thymic medulla the vein also is surrounded by a layer of epithelial cells but this cell layer is discontinuous. I conclude from these observations that the morphological appearances do not in fact suggest a blood-thymus barrier; such a barrier may have a functional basis but it does not have a morphological one.

FIG. 1 (Ito). Electron micrograph showing relationship of thymic parenchyma to a capillary. C = capillary. E = epithelial-reticular cell. M = mesenchymal-reticular cell. (× 2700).

Šterzl: We induced the state of immunological unresponsiveness—tolerance—in some experiments that have served as a basis for deducing that tolerance is the result of terminal exhaustive differentiation. Immunological unresponsiveness was induced in adult animals by long term immunization with bacterial antigens. Enhancement of antibody reaction was observed during the first month of daily injections, followed by a gradual depression of antibody formation (Šterzl,

J. [1954]. *In* Defence Processes in the Organism, Prague: Publishing House of the Czechoslovak Academy of Sciences; Šterzl, J. [1956]. *Folia biol.*, **2**, 129). Very similar results were obtained if excess doses of bacterial antigen were injected into neonatal animals (Šterzl. J., and Trnka, Z. [1957]. *Nature, Lond.*, **179**, 918). High doses enhanced antibody production in newborn animals in the first month of life. If the animals with an enhanced antibody response after birth were retested at four months, a significant decrease in this response was observed. We concluded from these experiments that induction of the state of tolerance precedes the immune reaction (Šterzl, J. [1962]. *In* Mechanisms of Immunological Tolerance, Proc. Symp. at Liblice, November, 1961, p. 109, ed. Hašek, M., Lengerová, A., and Vojtiškova, M. Prague: Publishing House of the Czechoslovak Academy of Sciences and New York: Academic Press).

We next used erythrocytes as antigen and measured the number of antibody-producing cells by the plaque technique. With this method we were able to estimate both γM and γG antibody-producing cells. If the plaques were developed by adding complement only γG antibody-producing cells were detected; if anti-γG serum and then complement were added, plaques were developed round the γG antibody-producing cells. The effectiveness of this method is illustrated in Fig. 1: in mice, 14 days after immunization, only 8 per cent of antibody-producing cells were developed with complement alone. The other 92 per cent of cells appeared only when anti-γG serum was added before complement (Šterzl, J., and Říha, I. [1965]. *Nature, Lond.*, **208**, 858). The antibody response to different amounts of antigen was tested by this method. Injection of increasing amounts of sheep erythrocytes into newborn rabbits or newborn sterile piglets resulted in an increase of antibody-forming cells as detected on the tenth day after immunization. We could not find any dose that led to immediate inhibition of antibody response. Groups of experimental animals to which various doses of sheep erythrocytes were given after birth were reinjected at the age of three weeks with the same antigen. The number of antibody-producing cells after the secondary stimulus was found to be significantly lower in the animals stimulated with maximum doses during the primary reaction, that is, in those animals that produced the maximum number of antibody-forming cells during the primary

reaction (Šterzl, J. [1966]. *Nature, Lond.*, **209**, 416; Šterzl, J. [1965]. *Communicated to* Information Exchange Groups, Nos. 5, 27 [Immunopathology]) (Tables I and II).

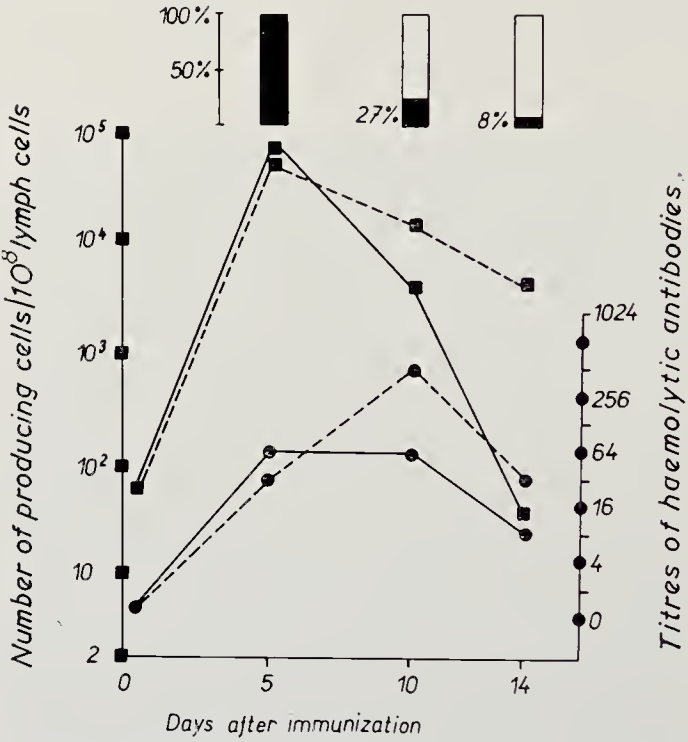

FIG. 1 (Šterzl). Dynamics of γM and γG antibody formation by individual cells in mice. Columns: black = percentage of cells detected by complement only, white = percentage of cells detected by anti-γG serum and complement. Graphs: number of cells detected by complement only—■——■, by anti-γG serum and complement—■---■. Titres of serum haemolytic antibodies detected by complement—●——●, by anti-γG serum and complement—●---●. (Šterzl and Říha, 1965. By permission.)

On the basis of these results we have assumed that acquired immunological tolerance may be considered as a result of the process of terminal exhaustive differentiation. The majority of, or perhaps all, immunologically competent cells are stimulated by excess antigen and differentiate directly into short-lived antibody-producing cells. After their death, only a small number of cells capable of responding to

specific antigen is available and thus a state of tolerance is produced. In contrast to this effect of high doses of antigen, small doses activate immunologically competent cells and induce proliferation of such activated cells and their descendants. Only a small proportion of these activated cells differentiate terminally into antibody-forming cells, and the rest provide the cellular basis for the secondary response.

TABLE I (Šterzl)

ANTIBODY FORMATION IN YOUNG RABBITS IMMUNIZED AFTER BIRTH WITH DIFFERENT DOSES OF SHEEP RED BLOOD CELLS AND REVACCINATED THREE WEEKS AFTER THE PRIMARY IMMUNIZATION

Dose of SRBC in primary immunization	*Average number of plaque-forming cells per 10^8 lymphoid cells*		
	8 days after primary stimulus	*5 days after secondary stimulus with 2×10^9 SRBC Developed by*	
	Complement only	*Complement only*	*Anti-γG serum + complement*
0	0	2,585	732
1 ml. 0·01 per cent SRBC (2×10^6)†	0	2,735	532
1 ml. 0·01 per cent SRBC (2×10^7)†	3	14,070	102,433
1 ml. 1 per cent SRBC (2×10^8)†	9	1,788	7,810
1 ml. 10 per cent SRBC (2×10^9)†	35	978	1,445
1 ml. concentrated SRBC (2×10^{10})†	46	66	166

†Number of cells. SRBC = sheep red blood cells. (From Sterzl, J. [1966]. *Nature, Lond.*, **209**, 416. By permission).

Woodruff: When we discussed all this at length at the recent (May, 1965) International Symposium on Immunological Tolerance at Locarno (unpublished), I pointed out that all the conditions Dr. Nossal has postulated for the production of tolerance could easily be reproduced in an *in vitro* model and yet it seems to be extremely difficult to produce tolerance *in vitro*. Dr. Nossal, why is this?

Nossal: I think there may be two answers to this question. Firstly, you will remember that N. A. Mitchison said at the Locarno Conference (1965, *loc. cit.*) that in occasional experiments he *had* found a suggestion that tolerance production occurred *in vitro*. I discussed complement-dependent lysis in my presentation merely because this is something simple that we know exists. What may be going on is

TABLE II (Šterzl)

ANTIBODY FORMATION IN STERILE PIGLETS IMMUNIZED AFTER BIRTH WITH DIFFERENT DOSES OF SHEEP RED BLOOD CELLS AND REVACCINATED THREE WEEKS AFTER THE PRIMARY IMMUNIZATION

Dose of SRBC in primary immunization	*Average number of plaque-forming cells per 10^8 lymphoid cells*		
	10 days after primary stimulus	*5 days after secondary stimulus with 2×10^8 SRBC Developed by*	
	Complement only	*Complement only (γM)*	*Anti-γG serum + complement (γG)*
0	0	0	0
10 ml. 0·02 per cent SRBC (2×10^7)†	0	0	0
10 ml. 0·1 per cent SRBC (2×10^8)†	0	0	0
10 ml. 1 per cent SRBC (2×10^9)†	22	2640	21,600
10 ml. 10 per cent SRBC (2×10^{10})†	89	1180	1660
10 ml. concentrated SRBC (2×10^{11})†	389	0	800

†Number of cells: SRBC = sheep red blood cells. (From Šterzl, J. [1966]. *Nature, Lond.*, **209**, 416. By permission).

almost certainly much more complex than this and may depend on pharmacologically active agents produced from eosinophils, from mast cells or from complex multichain types of reactions which we have not yet been able to reproduce in the test tube. To me this is an argument, but not a completely persuasive one.

Metcalf: What is the present state of the thymic inhibitory factor of Mowbray (Mowbray, J. F. [1963]. *Immunology*, **6**, 217)?

Nossal: I understand that J. H. Humphrey is trying to repeat some of Mowbray's work with material that Mowbray has supplied, but nothing has been published on this yet.

Miller: Dr. Nossal, do you think that the presence of antigen in thymus lymphoid cells is a *sine qua non* for tolerance induction, or that this may just reflect the level of antigen distribution in the body as a whole?

Nossal: I think both. You yourself have coined the word "epiphenomenon" for this. The thymus antigen concentration seems to hover around the critical level of one molecule per cell. If there are immunologically competent cells in the thymus, which there probably are, then they, together with all the other lymphocytes in the body, must be modified in the appropriate way for tolerance induction. This is important, but it is equally important that the cells in the diffuse cortex of the lymph nodes and in the white pulp of the spleen are also appropriately modified. There are so many phagocytic guardians to prevent this happening in the adult animal that the thymus may simply be one of many relatively antigen-protected sites. The reason that the final outcome of an immunological stimulus in adults is not immunological tolerance is that their opsonic and phagocytic systems are so active.

Burnet: Dr. Nossal, do you think that to produce tolerance by clonal deletion there must be direct contact of antigen or antigenic determinant with all the potential lymphoid cells; or do you favour the alternative theory that the production of antibody requires dendritic macrophages or similar cells?

Nossal: The answer to your first question evolves from an idea of your own: it seems to be necessary for contact to be made between the antigen and every cell that is, at that moment in time, forming the relevant antibody. In simple terms I envisage that the thymus directs a cell with a particular potentiality to start secreting whatever, at that particular moment, it has the ability to secrete. The answer to your second question is more complex. Undoubtedly γM-immunoglobulin synthesis can occur without the specialized macrophages. We know this from studies on the toad (Diener, E., and Nossal, G. J. V. [1966]. *Immunology*, in press), and the newborn rat (Williams, M., and Nossal, G. J. V., in preparation). In ontogeny and phylogeny, the development

of the follicular dendritic antigen-retaining web represents a sophisticated advance concomitant with the beginnings of γG-antibody synthesis and immunological memory. I think that probably the body decided that this was the most efficient way of mixing small quantities of antigen and lymphoid cells. It may represent, therefore, not an essential step for the most primitive types of immune responses but a necessary one for the sophisticated and fully developed phenomenon of immunity in the adult vertebrate.

TRAFFIC OF LYMPHOID CELLS IN THE BODY†

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For several years my colleagues and I have been using the T6 chromosome marker to investigate cellular movements in the mouse. In this context the expression "cellular movements" is intended to connote movements of cells that are capable of proliferation and that identify themselves by entering mitosis. In presenting an outline of the results of this work, I will give particular attention to evidence on the flow of cells into and out of the thymus and supplement the data pertaining explicitly to the mouse with limited information obtained from other species. Many of the results were obtained in collaboration and have not been published elsewhere.

EXPERIMENTAL DATA

THE T6 CHROMOSOME MARKER

T6 (Translocation 6) is an unequal radiation-induced reciprocal translocation between two of the shorter autosomes (Carter, Lyon and Phillips, 1955; Ford *et al.*, 1956). One of the products is a highly distinctive abnormal chromosome, readily distinguished in mitotic metaphase by its small size and characteristic tripartite appearance. By serial back-crossing to mice of the standard strain CBA/H and subsequent brother–sister mating Dr. Mary Lyon has established a new strain, homozygous for the translocation, on the CBA genetic background. This strain is designated

† Open lecture.

CBA/H-*T6T6*.† Dr. D. W. H. Barnes has demonstrated the effective immunological equivalence of the two strains by showing that reciprocal skin grafts are accepted and retained indefinitely. Effective physiological and genetic equivalence is shown by experiments in which bone-marrow cells of the two strains multiply at equal rates when injected into lethally irradiated host mice (Ford *et al.*, 1966).

THE FATE OF LYMPHOID CELLS INJECTED INTO IRRADIATED HOSTS

It is well known that injected lymphoid cells will not save a lethally irradiated mouse from death. However, if 10^5 bone-marrow cells are injected as well, the mouse will (usually) survive. Something can now be learned of the fate of the lymphoid cells by using, for example, CBA mice as hosts, T6T6 mice as donors of the bone-marrow cells and T6+ mice as donors of the lymphoid cells. Host cells would then have no marker chromosome, cells from the bone-marrow donors two, and cells from the lymphoid donors one (Ford and Micklem, 1963). All three cell types are presumptively equivalent; nevertheless, some experiments were set up in "balanced" form, that is with two parallel series of mice, one of which received T6T6 bone-marrow cells and T6+ lymphoid cells, and the other T6+ bone-marrow cells and T6T6 lymphoid cells. In no instance was this difference found to influence the outcome.

One striking result of these experiments is that if the hosts are killed in the second week after irradiation, the mitotic cells of the lymph nodes are found to be derived almost exclusively from the lymphoid donor and the mitotic cells of the bone marrow almost exclusively from the bone-marrow donor (Table I). This result has been obtained repeatedly and with lymphoid cells derived from both lymph nodes and thymus. It can therefore be made the basis of a functional test of one aspect of the behaviour of

† These two strains and their F_1 hybrid will be referred to as CBA, T6T6 and T6+ respectively.

TABLE I

MITOTIC CELLS IDENTIFIED IN LETHALLY IRRADIATED CBA MICE INJECTED WITH 10^7 LYMPH NODE CELLS OR THYMUS CELLS (T6T6) AND 10^5 BONE-MARROW CELLS (T6+)

		Numbers of mitotic cells identified in:					
		Bone marrow			*Lymph nodes*		
Number of mice	*Time after irradiation (days)*	*Bone-marrow donor*	*Lymphoid donor*	*Host*	*Bone-marrow donor*	*Lymphoid donor*	*Host*
5	7	861	21	11	4	96	15
4	10	484	31	3	3	220	9
7	14	1057	6	6	44	231	8

(Data of Micklem, Ford, Evans and Gray, unpublished.)

lymphoid cells. This differential representation of the two types of mitotic cell could be due either to *differential attachment* of circulating stem cells to different sites, or to *differential proliferation* in different sites or to some combination of both. We prefer to emphasize the first alternative and accordingly refer to the lymph-node-homing characteristics of lymphoid cells, but it is impossible to exclude the second alternative on the basis of this kind of experiment.

A second striking result of these experiments is the exceptionally small proportion of mitotic cells derived from the lymphoid donor that were identified in the thymus. This is in agreement with radioautographic experiments (Gowans and Knight, 1964). In general, very few cells derived from the lymphoid donor are found dividing in the bone marrow and, apart from the first ten days, even in the spleen. On the other hand, descendants of the injected bone-marrow cells quickly appear in bone marrow, spleen and thymus thereby confirming earlier results (Ford, Hamerton, Barnes and Loutit, 1956; Ford, Hamerton, Barnes and Loutit, 1957). Cells of myeloid origin are first identified in the lymph nodes somewhat later but rapidly increase to outnumber the cells derived from the lymphoid donor. Though histological evidence indicates that the first cells from the bone-marrow donor to divide in the lymph nodes may represent ectopic myelopoiesis, the later resumption of normal histological appearances by the lymph nodes strongly suggests that most of the immigrant cells of myeloid origin have transformed into lymphocytes. This supposition was supported by tests based on the differential behaviour of the lymphoid and myeloid cells just mentioned. These demonstrated that cells from the lymph nodes, but derived originally from myeloid donors, had acquired the capacity to home to the lymph nodes of secondary hosts in the same way as normal lymphoid cells. However, it cannot be inferred from this evidence that there is a generalized myeloid-lymphoid transformation in the lymph nodes of the irradiated

recipients. It could be postulated that there is a distinct class of stem cells present in normal bone marrow with a specifically lymphoid potentiality, and that these cells find their way to the lymph nodes of the recipients and multiply differentially, gradually replacing both the ectopic true myeloid cells *and* the cells derived from the lymphoid donor. Evidence to be presented later shows that there is indeed a class of cells in the bone marrow of the CBA mouse that has marked lymphoid potentiality (*vide infra*).

These results make it difficult to avoid the conclusion that cells of myeloid origin are more efficient than true lymphoid cells as progenitors of lymphoid tissue in radiation chimaeras. They also raise the possibility that, in the normal non-irradiated animal, there may be a progressive natural replacement of established lymphoid tissue by new lymphoid cells derived originally from a myeloid source.

The success of these experiments prompted a further series of tests of the same kind, designed to investigate the temporal changes in the properties of the cells derived from donor bone marrow in the *thymuses* of newly established syngeneic chimaeras. Lethally irradiated CBA mice were given 10^6 T6T6 bone-marrow cells and suspensions of thymocytes were prepared from them 16, 23 and 30 days later. The secondary hosts were also CBA mice and each received approximately 10^7 thymocytes plus 10^5 normal T6+ bone-marrow cells. The results of these experiments are given in Table II. At 16 days, cells derived from the bone-marrow donor were the overwhelmingly dominant component of the mitotic population of the thymus suspension, yet only one was identified in the nodes of the secondary hosts and none at all in the bone marrow. Evidently, at this time, the bone-marrow cells have lost the capacity to behave like normal myeloid cells but have hardly yet acquired the ability to home to lymph nodes. Seven days later, the lymph-node-homing capacity has appeared and, by 30 days, it is expressed more strongly though possibly not yet developed to its final level.

TABLE II

RETRANSPLANTATION OF THYMUS CELLS FROM RECENTLY ESTABLISHED SYNGENEIC CHIMAERAS

	Mitotic cells identified in:									
	Primary host		*Secondary host*							
	Thymus		*Bone-marrow*				*Lymph nodes*			
				Per cent from:				*Per cent from:*		
Time after irradiation (days)	*Total*	*Per cent from Primary donor*	*Total*	*Primary donor*	*Secondary donor*	*Primary or secondary host*	*Total*	*Primary donor*	*Secondary donor*	*Primary or secondary host*
16	72	87	389	0	99	1·0	99	1	26	73
23	60	95	287	0	99·3	0·7	88	33	48	19
30	35	97	350	0·3	99·4	0·3	73	68	25	7

Irradiated CBA males injected with 10^6 bone-marrow cells (T6T6). Groups of 3 or 4 killed 16, 23 and 30 days later and pooled thymus suspensions prepared; $(0{\cdot}6–2{\cdot}3) \times 10^7$ thymic cells plus 10^5 bone-marrow cells (T6+) then injected into irradiated secondary hosts (CBA) which were killed 7 to 14 days later. (Data of Micklem, Ford, Evans and Gray, unpublished.)

The results of a similar series of tests with cell suspensions prepared from the *lymph nodes* of recently established chimaeras are given in Table III. As in the tests of thymus cells, by 16 days after irradiation and injection the cells derived from the original bone-marrow donor have lost the capacity to contribute to myeloid regeneration. On the other hand, the capacity to home to lymph nodes is already strongly expressed, and barely increased in the later tests at 23 and 30 days.

TABLE III

RETRANSPLANTATION OF LYMPH NODE CELLS FROM RECENTLY ESTABLISHED SYNGENEIC CHIMAERAS

Time after irradiation of primary hosts (days)	*Mitotic cells identified in secondary hosts*					
	Bone marrow			*Lymph nodes*		
		Per cent from:			*Per cent from:*	
	Total	*Primary donor*	*Primary host*	*Total*	*Primary donor*	*Primary host*
16	405	0·2	—	130	48·5	—
23	325	0	0	137	50·4	17·5
30	107	0	0	135	51·9	19·3

Irradiated CBA males injected with 10^6 bone-marrow cells (T6T6). Groups of 10 killed 16, 23 and 30 days later and cell suspensions prepared from pooled lymph nodes. $(1{\cdot}7–2{\cdot}6) \times 10^6$ lymph node cells plus 10^5 bone-marrow cells (T6+) injected into irradiated secondary hosts, CBA (16-day series) or T6+ (23- and 30-day series). Secondary hosts killed 7 to 14 days after irradiation. (Data of Micklem, Ford, Evans and Gray, unpublished.)

Comparison of the results of these two experiments suggests that functional lymphoid cells derived from the bone-marrow inoculum appear in appreciable numbers earlier in the lymph nodes than in the thymus. If this conclusion can be sustained it would be difficult to reconcile with any hypothesis that regards the thymus as a *necessary* intermediary agent in the restoration of radiation-depleted lymphatic tissue by cells of myeloid origin. We cannot reach a definite conclusion from the present data because the two series of tests were performed with cells taken from two different batches of syngeneic chimaeras and these may not have been exactly comparable.

The loss of the capacity to contribute to myeloid regeneration in these unquestioned descendants of cells of the myeloid inoculum and their later (presumably) rather than concurrent acquisition of lymph-node-homing potential (at least where the cells concerned had been residing in the thymuses of their hosts) could be accounted for in two ways: either by a change of multipotential stem cells into proliferative cells of more limited potentiality, or by the selective proliferation (in lymphoid sites) of cells that had already become pre-adapted to this function while still part of the myeloid complex.

NEONATAL THYMECTOMY: THYMUS GRAFTING EXPERIMENTS

Miller (1962;1963) grafted thymuses taken from newborn mice into neonatally thymectomized mice when the latter were approximately one week old. Using the T6 chromosome marker method he reported that cells from the grafts could be identified later in the spleens of the host mice and also that the grafts, which can be assumed to undergo a period of involution followed by regeneration (cf. Carnes *et al.*, 1956), come to contain a very high proportion of dividing cells derived from the hosts.

The results of a similar experiment with the syngeneic CBA and T6T6 strains are shown in Table IV.

Cells from the graft were clearly able to reach the lymph nodes and multiply there in large numbers but none at all appeared in the bone marrow and, in contrast to Miller's finding, only very few in the spleen. On the other hand, these results confirm Miller's discovery that host cells multiply in the graft and suggest that they eventually become its sole supply of dividing cells.

The highest proportion of donor-type mitotic cells in the lymph nodes was observed in the first animals to be killed (in the fourth week of life). Thereafter the numbers of donor cells declined rapidly. A conceivable explanation of this decline is that the graft

continues to send out cells to the lymph nodes but that progressively more of them are of host type.

To test this possibility we carried out a retransplantation experiment (Harris and Ford, 1964). Mice were thymectomized at birth and grafted with thymuses from newborn animals, as before. The grafts remained in their primary hosts for between 21 and 127 days, after which they were transferred to fresh, neonatally thymectomized T6+ hosts. The secondary hosts were killed for cytological examination 14–55 days after the transfer.

TABLE IV

OBSERVATIONS ON NEONATALLY THYMECTOMIZED MICE GRAFTED SUBCUTANEOUSLY WHEN ONE WEEK OLD WITH THYMUSES FROM CHROMOSOMALLY DISTINCT NEWBORN MICE

Age of mice (days)	*Number of mice*	*Number of mitotic cells identified and per cent of donor cells in:*†		
		Graft	*Spleen*	*Lymph nodes*
20–26	7	283 (12)	785 (0·1)	461 (38)
31–35	3	214 (3·3)	293 (1·4)	335 (27)
55–88	9	656 (2·1)	724 (1·0)	617 (2·7)
90–132	6	686 (0)	603 (0·5)	719 (0·8)

† Figures in parentheses are the percentages.
(Data of Harris and Ford, 1963, and unpublished.)

Cells derived from the primary host were found in the lymph nodes of all of nine regrafted mice. The highest proportions observed were 47 per cent and 41 per cent respectively in animals with grafts that had remained a relatively long time in the primary host (70 days, 127 days) but a relatively short time in the secondary host (both 14 days). These results demonstrated that at least some of the cells of the primary host that entered the graft had acquired the capacity to behave like original thymic cells in their ability to move to, and enter mitosis in, the lymph nodes. What is the source of the host cells that repopulate the graft? Myeloid cells are the obvious candidates because of the barrier, already mentioned, to the entry of lymphoid cells into

the thymus. Another question that arises is whether there is a natural flow into the intact thymus of cells capable of settling and multiplying there. We now have evidence of three kinds that there is.

EVIDENCE FOR A NATURAL FLOW OF CELLS TO THE THYMUS

First, CBA mice were established with a pool of T6T6 cells in their femoral marrow. This was achieved by irradiating the hind quarters only—the forepart of the body, including the thymus, was shielded—and injecting T6T6 bone-marrow cells intravenously in the normal manner. T6T6 cells were identified in the thymuses of 37 out of 41 animals treated in this way, the highest proportion being 51 per cent in a mouse killed nine weeks after treatment.

Second, pairs of mice—each pair consisting of one CBA and one T6T6—were joined in parabiotic union by Dr. D. W. H. Barnes when they were four to five weeks old. The first two pairs were killed four and five weeks later and preparations were made from the bone marrow, spleen and lymph nodes as well as the thymus. The results demonstrated an appreciable movement of cells capable of undergoing mitosis from each animal into the thymus of its partner (Harris, Ford, Barnes and Evans, 1964).

These two pieces of experimental evidence support the idea that there is a natural replenishment of mitotic cells in the thymus from an extra-thymic site. However, they both involve surgical interference with the animal and this—through shock and the release of hormones from the adrenal cortex with consequent depletion of thymic tissue—might help to create the very conditions that would most favour detection of the effect we were looking for. The third line of evidence is not subject to this criticism. It is the consequence of an entirely natural process involving no experimental interference whatsoever.

Synchorial twinning is very common in marmoset monkeys. Benirschke and Brownhill (1962) demonstrated, by chromosomal

examination, the presence of male cells in the bone marrow of some adult females and female cells in the bone marrow of some adult males. Altogether they identified chimaerism of this kind in 6 out of 14 animals of five different species. It was easy to distinguish between the two types of cell because the males of all the species they examined had exceptionally small and easily recognizable Y chromosomes.

If our hypothesis applies to these animals, chimaerism should be detectable also in their thymuses. We obtained six young adult

TABLE V

NUMBERS OF MITOTIC CELLS IDENTIFIED IN THREE MARMOSET CHIMAERAS AND PERCENTAGES OF CELLS DERIVED FROM PRESUMPTIVE CO-TWIN

	Animal number					
	2		3		5	
Source of cells	*Total cells identified*	*Per cent from twin*	*Total cells identified*	*Per cent from twin*	*Total cells identified*	*Per cent from twin*
Bone marrow	209	24	137	55	117	37
Spleen	244	21	116	53	102	23
Thymus	98	14	117	80	64	17
Lymph nodes	173	13	112	52	35	11
Blood culture	17	12			8	36

(Data of Evans, Ford and Gray, unpublished.)

marmosets (*Oedipomidas oedipus*). Four of the six had both male and female cells in the bone marrow, thymus, spleen and lymph nodes and in cultured leucocytes of the peripheral blood. The number of mitotic cells identified in the different tissues of three of the chimaeras is given in Table V.

The information from the marmosets has now been supplemented by similar observations on a freemartin. Cells of both sexes were identified in bone marrow (52 per cent male), spleen (90 per cent male) and thymus (51 per cent male).

These results demonstrate a movement of cells that undergo mitosis into the thymus of both marmosets and freemartin, but

they do not show whether this is confined to foetal or to postnatal life, or whether it is a continuing process. All that can be said at present is that at *some* stage during the life of these two species, representing two widely different orders of mammals, there *is* a natural movement of cells into the thymus from a site or sites elsewhere in the body.

CELLULAR EXCHANGE IN PARABIOTIC MICE

We now have information on eight pairs of parabiotic mice joined at four to five weeks old and killed at various times up to

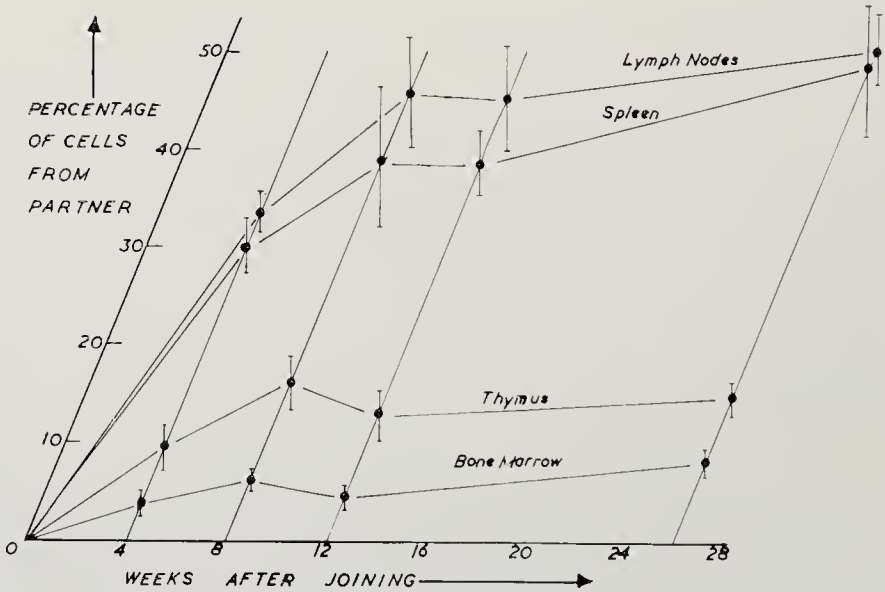

FIG. 1. Numbers of cells derived from partner expressed as mean percentage of total mitotic cells identified in 4 organs of 8 pairs of parabiotic mice (CBA and T6T6). Total cells identified: bone-marrow 3075, spleen 1758, thymus 3118, lymph nodes 2032. Standard errors calculated from the between-animals variance. (Data of Barnes, Evans, Ford and Gray, unpublished.)

thirty weeks later. The data on these mice are shown graphically in Fig. 1. We expected to find evidence of cellular exchange in the spleen and lymph nodes but we were surprised by the magnitude of this exchange. The level of exchange in the bone

marrow, on the other hand, was relatively low. The form of the curves indicates that the greatest part of the exchange must occur in the four weeks after the animals are joined. After this there is a close approach to the equilibrium level of 50 per cent of each type in spleen and lymph nodes, but only a small and doubtfully significant change in the thymus and bone marrow.

There is, therefore, extensive cellular exchange between the lymphoid tissues of parabionts. In a further series of parbiotic pairs we have demonstrated that this exchange is barely affected by thymectomy of both partners before they are joined (Barnes *et al.*, 1965). Parabiotic pairs with one partner thymectomized and the other left intact, however, present a different and more interesting situation. If the one remaining thymus continues to send out cells that have the capacity to reach the lymph nodes and attach and become the progenitors of lymphocytes, there should be an asymmetrical exchange between the partners: one would expect to identify more migrant cells in the lymph nodes of the *thymectomized* mouse than in the lymph nodes of its intact partner.

In eight out of ten such pairs the results were as anticipated, and four of the differences were very highly significant (Table VI). One of the two contrary differences (pair 84) was not statistically significant. The other (pair 71) was exceptional as the thymus of the intact mouse itself contained 63 per cent of mitotic cells from its partner. This was by far the highest level of exchange that we have encountered in the thymuses of any of our pairs. If this thymus had been releasing cells in the proportions observed, there would have been a greater flow of the *thymectomized* partner's cells to the lymph nodes of both animals, thereby increasing the level of apparent exchange in the nodes of the intact mouse and lowering it in the nodes of the thymectomized mouse. In fact, there is no significant heterogeneity between the proportions of the two types of mitotic cell in the one remaining thymus and the two sets of lymph nodes) $X^2_{[2]} = 1{\cdot}88$, $P > 0{\cdot}3$). The data from this pair are therefore not discordant with the hypothesis. The overall

TABLE VI

CELLULAR EXCHANGE IN PARABIOTIC PAIRS OF MICE: ONE PARTNER THYMECTOMIZED AT FOUR WEEKS, PAIRS JOINED ONE WEEK LATER

Pair Number	Time after joining (weeks)	Per cent of mitotic cells derived from partner: 1 Lymph nodes of intact mouse	2 Lymph nodes of thymectomized mouse	3 Thymus of intact mouse	Tests of equivalence of exchange between lymph nodes of partners ($\chi^2_{[1]}$)
69	4	33	44	8·5	5·46
74	4	32	40	5·2	3·45
72‡	4	23†	55	0·0	5·01
70	8	23	72	29·8	60·12
75	8	41	46	19·7	0·80
73	12	30	77	11·1†	69·55
71	12	56	43	62·7	6·86
84	15	58	48	20·9†	3·11
85	32	22	63	7·8	40·52
99	33	23	72	35·4	48·84

† Less than 100 cells scored.
‡ Incomplete thymectomy.
(Data of Barnes, Evans, Ford and Gray, unpublished.)

results of this experiment demonstrate that the thymus does influence the composition of the populations of mitotic cells of lymph nodes in parabionts.

An explanation of this finding in terms of a continuing cellular outflow from the thymus, though it accounts for the facts, is not the only possibility. The same result could occur if the thymus released into the circulation a short-lived substance one effect of which was to stimulate proliferation of lymphoid cells, and if the circulatory barrier between the members of a parabiotic pair was such that the equilibrium concentration of the thymic substance was appreciably higher in the intact mouse than in its partner. There would then be a greater proliferative stimulus to the lymphoid tissue of the intact mouse, with the expected consequence of a greater flow of cells *to* its partner than *from* its partner.

An experiment with ^{59}Fe-labelled erythrocytes (Barnes and Bungay, 1965) showed that there is very rapid cross-circulation between established parabionts. It could be argued that, however brief the delay and however short-lived the postulated thymic substance, the lymphoid tissue of the intact animal would always have the first contact with this substance and would therefore have the opportunity for an earlier and greater response than the lymphoid tissue of the thymectomized partner. We next need to know what the probability of effective reaction between the postulated thymic substance and its presumptive target would be at each passage in the circulation. If this is low in relation to the mean number of times the substance circulates within the intact animal before passing into the circulation of its partner, then the overall stimulus to each member of the pair would not be greatly different and a cellular explanation of our results would be favoured.

Another objection to the hypothesis of cellular outflow from the thymus is that some involution of lymphoid tissue may occur during the seven days between thymectomy and parabiosis so that at the time of joining there is already a relative deficiency of

lymphoid cells in the thymectomized partner. This deficiency might be expected to be rectified in part by a greater flow of lymphoid stem cells from the intact mouse.

On gross inspection at the time of killing, there were no evident differences in size between the lymph nodes or spleens of the intact member of each pair and the thymectomized partner. The mitotic activity of the lymph nodes, measured by the average number of scorable mitoses per preparation, was much the same in both members of each pair. There is thus no evidence for a difference in the amount of lymphoid tissue or in the level of its mitotic activity between the two members of a pair. We believe that these supplementary observations favour the cellular interpretation of the results of this experiment.

THE CONSEQUENCES OF PARABIOSIS FOLLOWED BY SEPARATION

In general there is good correlation between parabiotic partners in the level of exchange observed in corresponding organs. We therefore decided to separate a number of pairs after they had been joined together for eight weeks, kill one member of each pair immediately and use the data from it as a measure of the extent of exchange at that time, leaving the second animal to be killed four to twelve weeks later. The broad picture obtained from these comparisons is of a relatively small decline (16 per cent) in the proportion of migrant cells in the bone marrow during the period of separation, a somewhat greater decline in the lymph nodes (21 per cent) and a very considerable decline in the thymus and spleen (73 and 60 per cent respectively). The actual differences observed in the proportion of migrant cells in mitosis between corresponding organs of the first and second animal of each pair varied considerably. This was to be expected. The correlation at the time of separation would have been far from absolute and doubtless there would have been some variation between animals in the real rate of decline in the proportion of migrant cells after

separation. Such large differences would tend to obscure any temporal trend but our present data suggest that, after an initial sharp fall following separation, further decline in the proportion of migrant cells is small or non-existent.

It is conceivable that this result is the consequence of a natural process and reflects a relatively high level of movement of cells into the thymus and their expenditure by mitosis and differentiation. If this were so, and a new "steady state" level of migrant cells is established in the organ following separation, this level might be maintained by the persistent migrant cells in the bone marrow, which, we have found recently, have different properties from the resident population.

A DISTINCT COMPARTMENT OF PRECURSORS OF LYMPHOID STEM CELLS IN THE BONE MARROW

The evidence that migrant and resident cells in the bone marrow of parabionts have different properties is derived from lymph-node-homing tests. The results of one of these tests are given in Table VII. In the donor's bone marrow 80 resident (R) mitotic cells and eight migrant (M) cells were identified. This ratio was not significantly changed in the bone marrow of the two test mice, killed nine and ten days after treatment respectively. In the lymph nodes there was a most remarkable and highly significant increase in the numbers of descendants of the M cells compared with the numbers of descendants of the R cells. I have expressed this change numerically as an "enhancement factor" (though in the bone marrow of mouse number 3451 [see Table VII] this factor is a diminution and not an enhancement) by dividing the ratio of M to R cells identified in the test mice by the ratio of M to R cells identified in the original bone-marrow inoculum. An enhancement factor greater than unity indicates a relative increase of descendants of the M cells in the test mice; a factor of less than unity indicates a relative decrease.

TABLE VII

NUMBERS OF MITOTIC CELLS IDENTIFIED IN BONE MARROW OF ONE DONOR PARABIOTIC MOUSE AND IN BONE MARROW AND LYMPH NODES OF TWO IRRADIATED HOST MICE THAT HAD RECEIVED 10^6 BONE-MARROW CELLS FROM THE DONOR

		Bone marrow			*Lymph nodes*		
		Cells identified			*Cells identified*		
Mouse number	*Time after irradiation (days)*	*M (CBA)*	*R (T6T6)*	*Enhancement factor*	*M (CBA)*	*R (T6T6)*	*Enhancement factor*
3447	—	8	80	—			
3451	9	9	120	0·8	20	16	12·5
3453	10	17	155	1·1	228	14	162·9

M = migrant cells, R = resident cells. Enhancement factor = M/R (host mouse) ÷ M/R (donor parabiont)
(Data of Barnes, Evans, Ford, Gray and Ogden, unpublished.)

A series of tests of this kind was made with bone marrow from eight parabionts. Counts of mitotic cells were obtained from the bone marrow of 15 of the individual test animals and from the lymph nodes of 14. The results are shown in Table VIII. The bone marrow of the test animals gave enhancement factors ranging from 1·66 to 0. These either did not differ significantly from unity (twelve mice) or were significantly less than unity (three mice). This means that the ratio of M to R cells in the bone marrow of the secondary hosts did not become very

TABLE VIII

RATIOS OF MITOTIC CELLS DERIVED FROM MIGRATORY CELLS (M) AMD RESIDENT CELLS (R) IN THE BONE MARROW OF PARABIONTS OBSERVED IN THE BONE MARROW AND LYMPH NODES OF IRRADIATED HOST MICE

Ratio M/R	*Bone marrow*	*Lymph nodes*
Number of observations	15	14
Significantly greater in host than donor	0	7
No significant difference between host and donor	12	7
Significantly less in host than donor	3	0
Enhancement factor:		
Maximum	1·66	162·9
Minimum	0·0	3·6

Enhancement factor = M/R (host mouse) ÷ M/R (donor parabiont).
(Data of Barnes, Evans, Ford and Ogden, unpublished.)

different from the ratio in the marrow of the donor parabiont; if anything there was a slight decrease of M cells in the secondary hosts.

The striking relative increase of M cells in the lymph nodes of the test mice (Table VII) was confirmed in the rest of the series (Table VIII). The enhancement factor was significantly greater than unity in 7 of the 14 tests; in the remainder insufficient mitoses were identified to establish a significant difference. The conclusion, nevertheless, is clear: the cells that migrate from one parabiont into the bone marrow of the other, when compared with the

resident myeloid cells, have a greatly enhanced capacity to find and/or multiply in the lymph nodes of irradiated recipients. This suggests that there is a distinct component of the complex of normal myeloid cells that is characterized by mobility and a propensity for settling and multiplying in lymphoid tissue.

CONCLUSIONS

The interpretation of these results has been to some extent dependent on the assumption that our counts of cells in mitosis are truly representative of the proliferating cell populations in the tissues or organs examined. I believe that they are on the basis of tests to be described elsewhere.

If our counts are representative there is still much to be done before all our findings can be welded into a fully coherent picture of the cellular movements between the various sites of myeloid and lymphoid tissue in the mouse. A major difficulty is that we are dealing with the statistical properties of whole cell populations that are likely to be complex aggregates of several components with different functions and different responses to various experimental situations. Even so, we may hope that our increasing means of "physiological dissection" will eventually allow us to resolve the heterogeneity.

There is now good evidence that there is a movement of cells *into* the thymus from elsewhere in the body, probably from myeloid tissue, and that this movement may be widespread in mammals. Evidence for a natural movement *from* the thymus is not decisive, since it rests largely on grafting experiments, though it seems unlikely that so specific a property could be entirely an experimental artefact. There may also be a movement directly from myeloid tissue into the lymph nodes without the intervention of the thymus.

There is evidence that in the bone marrow there is a differentiated group of cells which is more mobile than the remaining

myeloid cells and potent as precursors of lymphoid stem cells in the lymph nodes. It is not yet known if these cells move to the lymph nodes directly or by way of the thymus.

Finally, there is the difficult question of whether any of our data provide proof of transformation in the properties of cells that have moved from one location to another, or whether all our findings can be explained on the basis of the selective proliferation of cells whose distinguishing properties were acquired at other sites. I think that transformation is probable for the myeloid cells that repopulate the lymph nodes of radiation chimaeras, but this is not yet proven. For the thymus, however, the facts that (a) myeloid cells proliferating in this organ lose the capacity to repopulate bone marrow and (b) normal thymic cells and cells from other sources that have resided in the thymus have a particular capacity for settling and proliferating in lymph nodes and will not return to the thymus, seem to me to be decisive. In other words, I believe that descendants of the cells that enter the thymus become differentiated on lines imposed by it. I do not think it remarkable that this should be so.

ACKNOWLEDGEMENTS

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THE THYMUS IN RELATION TO THE DEVELOPMENT OF IMMUNOLOGICAL CAPACITY

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Experiments with neonatally thymectomized mice have established the notion that the thymus is essential for the normal development and function of the lymphoid system (Miller, 1961, 1962*a*, 1964). The manner in which the thymus plays this essential role has not been clearly established. Several hypotheses have been formulated: the thymus may commit immigrant stem cells to differentiation exclusively along lymphoid pathways; it may, by means of a humoral factor, ensure that cells become capable of reacting immunologically, or it may exert a censorship action in order to eliminate self-reacting clones of cells. These hypotheses have been discussed elsewhere (Miller, 1965*a*).

INFLUENCE OF AGE ON THYMUS-DEPENDENT IMMUNOGENESIS

Thymectomy in adult mice or rats has been associated with a diminution in the population of lymphocytes in the peripheral blood, thoracic-duct lymph, lymph nodes and spleen (Metcalf, 1960; Bierring, 1960). However, no significant defects in the capacity for rejecting allogeneic skin homografts or for producing serum antibodies to some antigens have been reported in mice (Miller, Doak and Cross, 1963), guinea pigs (Fichtelius, Laurell and Philipsson, 1961) or rabbits (Harris, Rhoads and Stokes,

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1948; MacLean *et al.*, 1957) thymectomized in adult life and challenged within 1 to 2 months after thymectomy. This is in striking contrast to the severe immunological defects which occur following neonatal thymectomy (Miller, 1961, 1962*a*; Good *et al.*, 1962). These observations have suggested that the thymus is essential to establish an immunological system during the development of the animal. Evidence that the function of the thymus in initiating immunogenesis is not necessarily restricted to early life has, however, been produced. Adult mice, thymectomized and subsequently exposed to total-body irradiation, have shown severe deficiencies in some immunological functions (Miller, 1962*b*; Globerson, Fiore-Donati and Feldman, 1962; Miller and Osoba, 1963; Miller, Doak and Cross, 1963) indicating that the presence of the thymus in the adult is still essential if immune mechanisms are to be re-established when the immunological apparatus has been damaged or destroyed. Furthermore, it has been shown that thymectomy in an adult, immunologically tolerant of a particular antigen, has prevented the reappearance of reactivity with respect to that antigen (Claman and Talmage, 1963). The thymus in the adult thus appears to be required for the correction of specific immunological defects.

Further evidence for a continuing immunological function of the thymus through adult life has been obtained. Inbred CBA mice and $(Ak \times T6)F_1$ mice were thymectomized at birth or at 2 to 3 months of age. A sham operation involving thoracotomy but not removal of the thymus was performed in litter mates. The mice were challenged at various intervals up to 2 years after thymectomy with 0·3 ml. of a 5 per cent suspension of sheep erythrocytes in saline. From 4 to 6 days after challenge the mice were killed and their spleens removed to assay the number of antibody-plaque-forming cells per million spleen cells according to the technique of Jerne, Nordin and Henry (1963). Routine histological studies were performed on the thymus area in all thymectomized mice to ensure that thymectomy had been

complete. Mice with thymus remnants were discarded from the experiments. The spleens of normal and thymectomized mice aged between 1 week and 4 months and not challenged with sheep erythrocytes usually gave less than one antibody-plaque-forming cell per million spleen cells. In CBA mice, sham-thymectomized at birth and challenged at 10 days of age, the peak level of antibody-plaque-forming cells was 106 ± 6 and in $(Ak \times T6)F_1$ mice similarly treated it was 204 ± 27 (Table I). The corresponding peak levels for neonatally thymectomized mice were 4 ± 2 and 5 ± 2 respectively. These differences are highly statistically significant ($P < 0{\cdot}001$). Neonatal thymectomy thus practically inhibited the *development* of the capacity to produce antibody-plaque-forming cells in response to an injection of sheep erythrocytes. This impairment was evident in clinically-healthy suckling baby mice, the growth rate of which was identical to that of sham-thymectomized litter mates. When the mice were thymectomized or sham-thymectomized at 2 months of age and challenged with sheep erythrocytes 1 to 2 months later, there was no difference in the capacity of control and experimental mice to produce antibody-plaque-forming cells, the peak levels being the same in both groups. However, thymectomized mice challenged nine or more months after the operation produced less antibody-plaque-forming cells than controls (Table I). Similar results have been reported elsewhere after challenging with sheep erythrocytes and other antigens (Taylor, 1965; Metcalf, 1965; Miller, 1965*b*).

It can be concluded, therefore, that thymectomy of adult mice is associated with a decline in immunological capacity which becomes significant after a period of 6 to 9 months. The thymus must thus continue to function during adult life to ensure the *maintenance* of an adequate pool of immunologically competent cells. Thymectomy in the adult, unlike thymectomy in the newborn, has no immediate effect upon immunological capacity, presumably because an adequate pool of competent cells has

TABLE I

INFLUENCE OF AGE AT THYMECTOMY ON THE PRODUCTION OF ANTIBODY-PLAQUE-FORMING CELLS IN THE SPLEEN

Strain	*Operation*	*Age at operation*	*Age at immunization*	*Number of mice*	*Number of mice† with following number of antibody-plaque-forming cells per million spleen cells*					
					0–20	21–100	101–300	301–500	501–800	801–1200
$(Ak \times T6)F_1$	Sham-thymectomy	Birth	10 days	8			8			
	Thymectomy			10	10					
	Sham-thymectomy	2–3 months	4 months	5						5
	Thymectomy			6						6
	Sham-thymectomy	2–3 months	9 months	6						6
	Thymectomy			8				6	2	
CBA	Sham-thymectomy	Birth	10 days	8		2	6			
	Thymectomy			6	6					
	Sham-thymectomy	2–3 months	4 months	4						4
	Thymectomy			4						4
	Sham-thymectomy	2–3 months	18 months	5				1	4	
	Thymectomy			7	1		4	1	1	
	Sham-thymectomy	2–3 months	24 months	3				2	1	
	Thymectomy			3	1	2				

† Killed at peak titre after immunization.

already been constructed. Only when the pool becomes depleted, owing to the limited life-span of its cells, do immunological defects with respect to newly encountered antigens become evident.

REGENERATION OF THYMUS GRAFTS

The immunological defects of thymectomized animals have been corrected by injecting suspensions of cells obtained from the lymph nodes and spleens of adult animals of the same inbred strain (Miller, 1964; Miller, Osoba and Dukor, 1965; Cross, Leuchars and Miller, 1964; Trainin, Law and Levey, 1965). Presumably the thymectomized animals in these experiments were supplied with an already constructed pool of immunologically competent cells which could respond as adequately in their new hosts as in their original normal hosts. To study the relationship between the thymus and this pool of immunologically competent cells experiments were performed on thymectomized mice grafted with thymus tissue. Strain combinations were used, the cells of one strain being characterized at metaphase by the presence of marker chromosomes (T6). It was found that the immunological defects of mice thymectomized at birth (Miller, 1961, 1962*a*, 1962*c*) or thymectomized and irradiated in adult life (Miller *et al.*, 1964; Leuchars, Cross and Dukor, 1965) could be adequately corrected by grafting syngeneic or allogeneic thymus tissue. To understand the mechanism of action of thymus grafts a detailed study of the histological and cytological events taking place in the thymus implants and in the lymphoid tissues of the host was undertaken (Dukor *et al.*, 1965). These events are summarized here for syngeneic, allogeneic, and *in vitro* irradiated thymus tissue grafted to normal (Table II) or pretreated (Table III) hosts.

In normal hosts grafted with syngeneic thymus tissue the implant regained normal thymus architecture within a week.

TABLE II

REGENERATION OF SYNGENEIC, ALLOGENEIC AND SYNGENEIC IRRADIATED THYMUS TISSUE IN NORMAL HOSTS†

Time after grafting	*Syngeneic*	*Allogeneic*	*Syngeneic irradiated* (500–2000 r.)
Day 1–2	Necrotic centre; viable lymphocytes and epithelial-reticular cells in periphery	Necrotic centre; viable lymphocytes and epithelial-reticular cells in periphery	Necrotic centre; viable epithelial-reticular cells at periphery; no lymphocytes
Day 3–4	Intense mitotic activity of *donor-type* cells in lymphoid rim	Lymphoid cells disappear from epithelial-reticular framework	Implant consists only of epithelial-reticular cells
Day 5–6	Cortical architecture restored	No lymphoid differentiation; "small-lymphocyte-like cells" and myeloid foci appear	No lymphoid differentiation; "small-lymphocyte-like cells" and myeloid foci appear
Day 7–11	Normal thymus architecture restored	Invasion by typical small lymphocytes and histiocytes; cell destruction evident	Large lymphocytes appear among other cell types
Day 11–15	*Host-type* dividing cells appear in the graft	Fibrous scar (sometimes a few scattered epithelial-reticular cells remain)	Mitotic activity evident in lymphoid cells; dividing cells are of *host type*; medium and small lymphocytes appear
Day 15–20	Progressive and complete replacement of mitotic cells by *host-type* cells	Fibrous scar	Normal thymus architecture restored

† Data from Dukor *et al.* (1965).

TABLE III

REGENERATION OF SYNGENEIC, ALLOGENEIC AND SYNGENEIC IRRADIATED THYMUS TISSUE IN PRETREATED HOSTS

Treatment of host	*Syngeneic*	*Allogeneic*	*Syngeneic irradiated* (500–2000 r.)
Neonatal thymectomy*	Regenerates as in normal host	Regenerates as syngeneic graft	Regenerates as in normal host
Adult thymectomy*	Regenerates as in normal host	Regenerates and rejected as in normal host	Regenerates as in normal host
Adult thymectomy and irradiation*	Regenerates as in normal host	Some lymphoid differentiation in some grafts; rejection eventually occurs	Regenerates as in normal host
Irradiation* (sublethal or lethal + bone marrow)	Regenerates as in normal host	Some lymphoid differentiation takes place; rejection eventually occurs	Regenerates as in normal host†
Lethal irradiation (no bone marrow)	Some lymphoid differentiation in some grafts	Epithelial-reticular framework totally devoid of lymphoid and myeloid elements‡	Epithelial-reticular framework totally devoid of lymphoid and myeloid elements§

* Data from Dukor *et al.* (1965).
† See Figs. 1 and 2.
‡ See Fig. 3.
§ See Fig. 4.

The thymus graft was composed of donor-type cells during these first 10 to 15 days but gradual and complete replacement of the mitotic lymphoid cells by host-type cells occurred during the next week. The regeneration pattern of these syngeneic grafts was unaffected by previous thymectomy or irradiation of the host. In contrast to syngeneic grafts, allogeneic thymus tissue

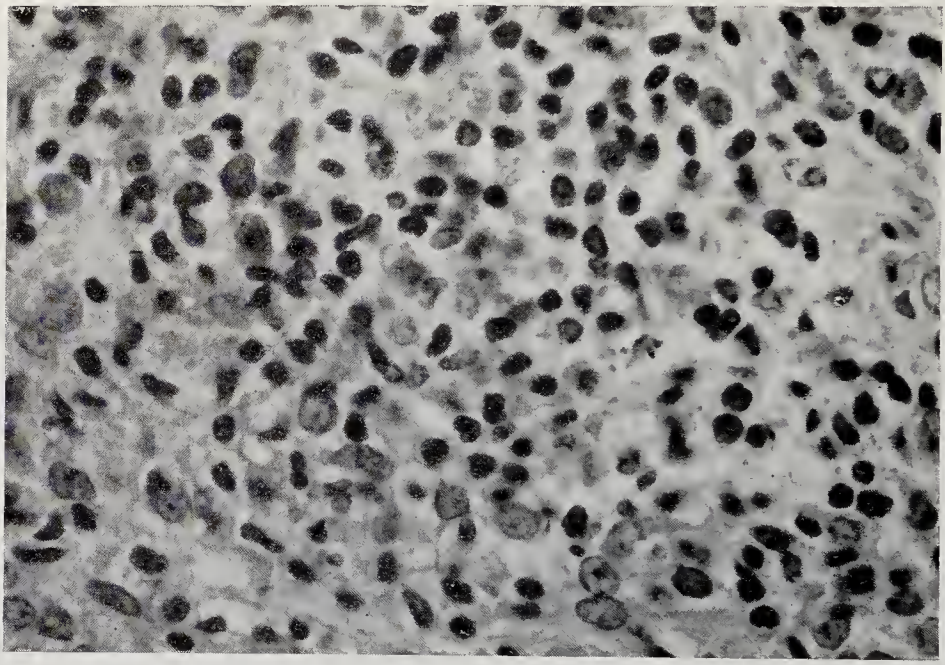


FIG. 1. Thymus tissue irradiated *in vitro* with 2000 r. and residing for 7 days under the renal capsule of normal syngeneic host. Note numerous scattered small cells with nuclei showing an extremely dense chromatin structure. (× 675).

lost the lymphocytes surviving in the peripheral rim and failed to undergo lymphoid differentiation. Instead, myeloid foci were evident and the graft was eventually rejected after having been infiltrated by typical small lymphocytes and histiocytes. Normal lymphoid differentiation occurred in allogeneic implants grafted to neonatally thymectomized mice. Irradiation of the host only delayed, and did not prevent, rejection of the graft. Thymus tissue irradiated *in vitro* with 500 r. or 2000 r. was depleted of

lymphocytes and composed of only epithelial-reticular cells during the first 4 days after grafting. Between 4 and 8 days, cells resembling small lymphocytes, but with practically no cytoplasm and a nucleus containing an extremely dense chromatin structure, first appeared in capillaries and then scattered in the epithelial-reticular framework (Fig. 1). These cells were considered to be

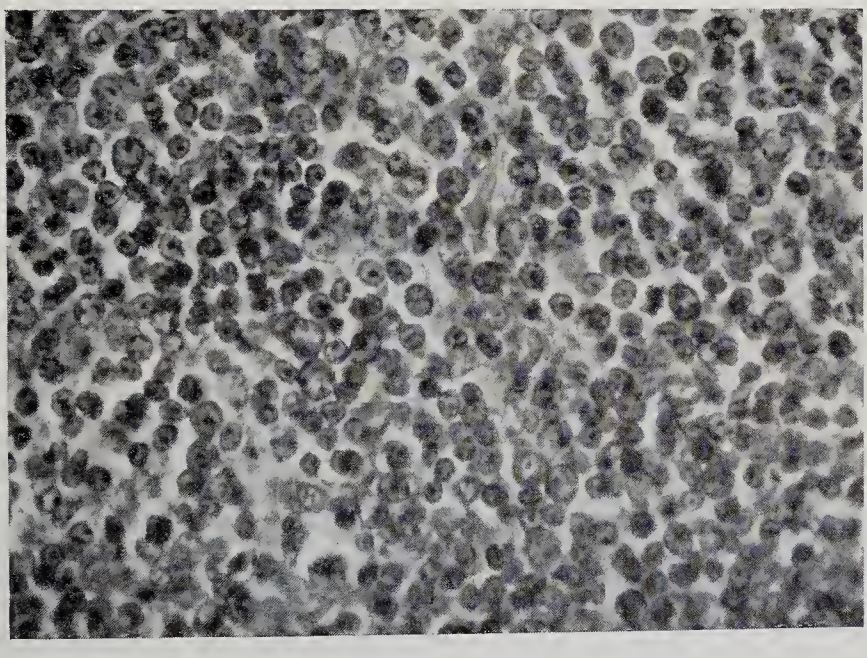


Fig. 2. Typical thymus lymphocytes. (× 675).

different from typical thymus small lymphocytes (Fig. 2) and were referred to as "small-lymphocyte-like cells" (Dukor *et al.*, 1965). As in allogeneic implants, myeloid foci appeared but, in contrast to allogeneic grafts, large lymphocytes and dividing lymphoid cells of host-type were seen by 7 to 8 days. By day 15 to 20 normal thymus architecture was restored in grafts that had been preirradiated with 500 r. as well as in those exposed to 2000 r. The origin of the "small-lymphocyte-like cells" and myeloid cells seen in allogeneic and irradiated thymus implants is

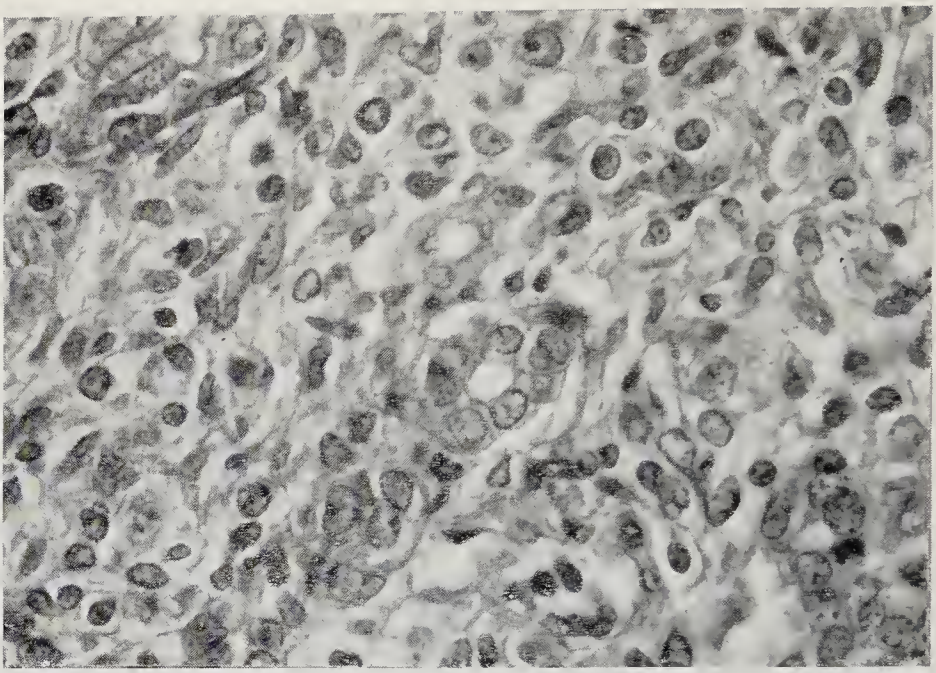


FIG. 3. Allogeneic thymus graft, 7 days after implantation into lethally irradiated host not protected with bone marrow. Note preponderance of epithelial-reticular cells and absence of small cells with dense chromatin structure. (× 675).

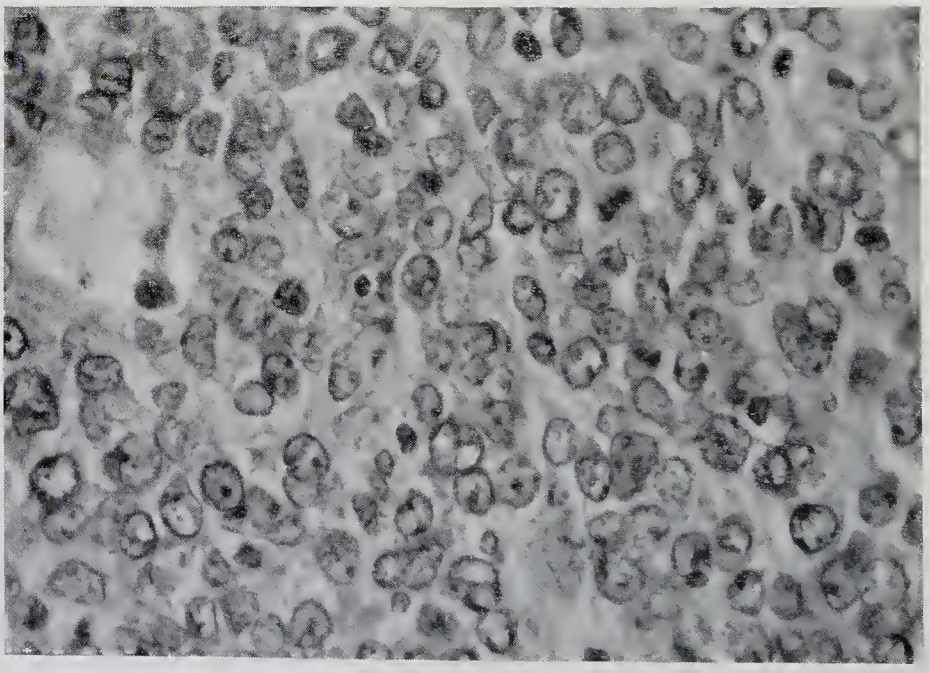


FIG. 4. Thymus tissue irradiated *in vitro* with 2000 r. and residing for 7 days under the renal capsule of lethally irradiated syngeneic host not protected with bone marrow. Note preponderance of epithelial-reticular cells and absence of small cells with dense chromatin structure. (× 675).

considered to be from the host rather than from the graft since these cells were absent in implants grafted to lethally irradiated hosts not injected with bone marrow (Figs. 3 and 4).

EFFECT OF THYMUS GRAFTS ON IMMUNOLOGICAL CAPACITY

The mechanism by which grafts restore immune capacity in thymectomized hosts is not known. The graft may have to provide cells which become competent after migrating to lymph nodes and spleen; if this is so the presence of thymus-derived cells in such organs would be expected in animals in which immune capacities have been restored. On the other hand, the implant may have to attract host lymphoid-precursor cells to enable them to differentiate in its environment to cells capable of reacting immunologically once they settle in lymph nodes and spleen: if this is the case restoration of immunological capacity would not be expected to occur before host cells could have migrated into the graft, an event which begins between 10 and 15 days after grafting. Finally, the implant may simply have to provide a humoral factor which would enable the differentiation of host lymphoid cells to immunologically competent cells. In this case, traffic of cells to and from the graft would not be essential. Evidence for the existence of a humoral factor has already been obtained in studies employing thymus tissue in Millipore diffusion chambers (Osoba and Miller, 1963, 1964; Law *et al.*, 1964). In order to determine the possible mechanisms by which thymus grafts restore immune capacities experiments were performed on neonatally thymectomized hosts bearing thymus tissue for limited periods. Syngeneic and allogeneic thymus grafts, and thymus tissue irradiated *in vitro* with 500 r. and 2000 r. before implantation, were used. The implants were excised after various intervals and the immunological capacity of the thymectomized hosts was determined.

Sham-thymectomized mice injected with sheep erythrocytes produced an average peak titre of 220 ± 25 (S.E.)† plaque-forming cells 4 days after immunization at 28 to 31 days of age. Thymectomy at birth dramatically reduced this peak to $9 \pm 4{\cdot}8$ plaque-forming cells per million spleen cells (Table IV). The difference between the peak titres of thymectomized and sham-thymectomized mice was highly statistically significant ($P > 0{\cdot}001$).

The presence for 10 days (from 7 to 17 days of age) of a non-irradiated thymus graft from either the parental strain, T6, or an unrelated strain, C3H, restored the capacity of thymectomized mice to produce a peak titre, 4 days after immunization at 28 to 31 days of age, of 72 ± 18 and 47 ± 11 plaque-forming cells per million spleen cells, respectively. The difference between these peaks and the peak level of $9 \pm 4{\cdot}8$ recorded in thymectomized controls is statistically significant ($P < 0{\cdot}01$).

The presence for 10 days of a T6 thymus graft, irradiated *in vitro* with 500 r., also restored to some extent the capacity of thymectomized mice to form plaque-forming cells: the peak level in this group of mice was 36 ± 8 and the difference between this and the peak level of thymectomized controls is statistically significant ($P < 0{\cdot}01$). By contrast, the presence for 10 days of a T6 thymus graft, irradiated *in vitro* with 2000 r., failed to restore immune capacity in thymectomized recipients.

The effects of thymectomy and thymus grafting on the survival of C3H (H2k) and C57BL (H2b) skin homografts on $(Ak \times T6)F_1$ (H2k) were also investigated. All the mice sham-thymectomized at birth rejected the foreign skin grafts within 20 days in marked contrast to mice subjected to total thymectomy at birth. The latter failed to reject the grafts, which grew luxuriant tufts of hair and were present for 2 months or more. Most thymectomized mice bearing normal T6 thymus grafts for 14 days had rejected C3H and C57BL skin grafts before 25 days. The presence

† Numbers after the peak-titre values in this and subsequent paragraphs refer to standard errors.

TABLE IV

EFFECT OF THYMECTOMY AND THYMUS GRAFTS ON THE RESPONSE OF MICE TO SHEEP ERYTHROCYTES AND SKIN HOMOGRAFTS

Treatment	*Peak number and standard error of antibody-plaque-forming cells*	*Mice rejecting skin graft before 25 days*†	
		C3H	*C57BL*
Sham-thymectomy at birth	220 ± 25	6/6	6/6
Thymectomy at birth	9 ± 4·8	0/6	0/6
Thymectomy at birth. T6 thymus graft for 10 days	72 ± 18	4/6	5/6
Thymectomy at birth. C3H thymus graft for 10 days	47 ± 11	0/7	4/7
Thymectomy at birth. T6 thymus graft (irradiated with 500 r.) for 14 days	36 ± 8	5/8	6/8
Thymectomy at birth. T6 thymus graft (irradiated with 2000 r.) for 14 days	8 ± 4·1	0/6	0/6

† Numerator = number of mice rejecting skin graft within 25 days.
Denominator = number of mice grafted.

of a C3H thymus graft for 2 weeks enabled 4 out of 7 neonatally thymectomized (Ak × T6)F_1 mice to reject, within 25 days, C57BL skin but not C3H skin; the latter was still present after an observation period of 140 days.

Thymectomized mice, bearing thymus tissue for 2 weeks and irradiated *in vitro* with 2000 r., failed to reject C57BL and C3H skin grafts. Irradiation of thymus tissue with 2000 r. thus impaired its capacity to restore homograft immunity. On the other hand, irradiation with 500 r. did not significantly impair this capacity: most mice bearing such thymus grafts for 2 weeks rejected C3H and C57BL skin grafts within 25 days.

TABLE V

CYTOLOGICAL ANALYSIS OF LYMPH NODES OF THYMECTOMIZED AND THYMUS-GRAFTED MICE

T6 implant in thymectomized (Ak × T6)F_1 host	*Percentage of thymus-derived cells in*		
	Unstimulated node	*Regional node (graft rejection)*	*Node draining area of trauma*
Non-irradiated thymus graft	3·2	20·7	8·3
Thymus graft pretreated with 500 r.	0	0	0

Number of cells scored in each group varied from 250 to 491. Cells were counted 4 to 7 weeks after grafting.

The results of a cytological analysis of the lymph nodes of (Ak × T6)F_1 mice, thymectomized at birth and grafted at 1 week of age with normal or irradiated (500 r.) parental-strain thymus tissue, are shown in Table V. The grafts were excised 7 to 10 days after implantation and the mice that had received normal and irradiated thymus tissue were further subdivided into 3 groups. One group received no further treatment and was sacrificed for cytological examination of lymph nodes at 7 to 9 weeks of age. Mice in another group were grafted with foreign skin (C57BL) at about 5 weeks of age and sacrificed at the height of the skin graft rejection for examination of the regional lymph node. Mice in the third group had a piece of skin excised but no

skin graft and were killed 7 days later for examination of the regional lymph node. The great majority of the dividing cells in the lymph nodes of mice which received a normal T6 thymus graft and no further treatment were of host-type: only 13 thymus-derived cells were seen among 410 metaphases scored. Not one thymus-derived cell was found in the lymph nodes of the corresponding group of mice receiving irradiated thymus grafts. At the height of skin graft rejection in the mice receiving the normal thymus graft 20·7 per cent of thymus-derived cells were found in the regional lymph node. Mice subjected to trauma *per se* had only 8·3 per cent thymus-derived cells in the regional lymph nodes. No thymus-derived cell was ever found in the lymph nodes of the groups of mice subjected to trauma or rejecting skin homografts and bearing irradiated thymus grafts.

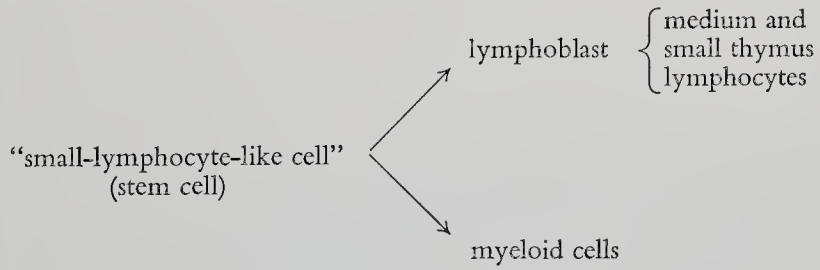
DISCUSSION

The regeneration of syngeneic thymus grafts was effected entirely by cells present in the implant at the time of grafting and hence by cells in the outer rim which had escaped destruction. A complete replacement of the dividing cell population (mainly lymphocytic) of the fully regenerated implant by host-type cells occurred during the third week after grafting. These observations are in agreement with those reported previously from this and other laboratories (Miller, 1962*d*; Miller and Osoba, 1963; Harris and Ford, 1964; Metcalf and Wakonig-Vaartaja, 1964). Since donor-type lymphocytes disappear from the implant it appears that the thymus has only a relatively small compartment of stem cells with limited capacities for self-renewal. The fate of the "mature" thymus small lymphocyte is debatable and only very occasionally was a small percentage of thymus donor-type elements found in the dividing cell population of the lymph nodes. However, the proportion of these cells was increased in nodes draining an area of trauma and considerably increased in nodes regional to a skin homograft at the height of

the rejection. Direct evidence has thus been obtained for the migration of cells from a thymus graft (present for only 7 to 10 days) to sites of immunological activity. In parallel investigations on thymectomized irradiated mice bearing thymus implants, it has also been found that the percentage of thymus-derived cells increases considerably in the spleen and lymph nodes following antigenic stimulation (Leuchars, Davis and Koller, 1966). These cytological results might at first sight suggest that the correction of immunological defects in thymectomized mice depends exclusively on cells, produced by the thymus implant, which migrate out and respond to an antigenic stimulus by proliferation in the lymphoid tissues. The significance of the increase in the proportion of thymus-derived cells in such areas under such conditions has not, however, been elucidated. It does not necessarily follow that these cells are directly or indirectly involved in antibody production or homograft rejection. Experiments in which thymus-derived cells were inhibited by specific iso-immunization of the host gave results which suggested that the presence of these cells was not essential for immunological reactivity (Leuchars, Davis and Koller, 1966). The question of the significance of thymus-derived cells in the lymph nodes of antigenically stimulated animals thus remains open.

The regeneration of thymus tissue irradiated *in vitro* with 500 r. or 2000 r. differed from that of normal thymus in several important respects: no lymphoid cells escaped destruction and the implant was composed solely of epithelial-reticular cells during the first 3 to 4 days; no donor cells were found dividing in the implant and only after 11 days, at a time when the grafts were becoming lymphoidal, were host-type dividing cells detected. These observations are consistent with the notion that thymus lymphoid cells (in postnatal life) are not derived directly from epithelial-reticular cells but are the progeny of cells entering the implant from the circulation. One of the sources of the invading host cells is likely to be the haemopoietic tissue of the bone

marrow. It has been shown that marrow-donor-type cells can settle in irradiated thymus (Ford and Micklem, 1963) and repopulate thymus implants in lethally irradiated animals (Dukor *et al.*, 1965). In the present study no lymphoid elements were ever found in irradiated thymus grafted to lethally irradiated hosts not protected with bone marrow. The identity of the cell type or cell types which migrate to the thymus to give rise to thymus lymphocytes is unknown. The marrow contains a high proportion of cells with morphological features similar to those of small lymphocytes but without their functional characteristics (Cross, Leuchars and Miller, 1964). It has been suggested that such cells may have stem-cell properties (Cudkowicz, Bennett and Shearer, 1964). When there was no evidence of lymphopoiesis and when no typical thymus lymphocytes were present in allogeneic and irradiated thymus implants, "small-lymphocyte-like cells" appeared. They did not appear in such implants grafted to lethally irradiated hosts which had not been protected with bone marrow. In normal hosts, or in irradiated marrow-protected hosts, the appearance of these cells was soon followed by the appearance of myeloid cells and, in the case of irradiated thymus tissue, large lymphocytes and lymphopoiesis were evident 4 to 8 days later. The implication of these observations is that there is, in marrow tissue, a cell type with morphological resemblances to a small lymphocyte which can migrate to thymus tissue to give rise either to myeloid cells or to thymus lymphocytes, depending on the circumstances. Hence the following sequence of events may take place:



It is emphasized, however, that critical experiments are yet to be performed in order to prove or disprove the existence of these pathways.

The presence, for 10 to 14 days, of thymus tissue irradiated *in vitro* with 2000 r. failed to restore the immunological defects of neonatally thymectomized mice. It could be argued that this is because no typical thymus small lymphocytes appear in irradiated grafts during the first two weeks; the implants regain their complement of lymphocytes only after the immigration of host lymphoid-precursor cells, which then differentiate and proliferate to form typical thymus lymphocytes. Only after these become available for distribution to other tissues might the reversal of immunological inadequacies become evident. This idea cannot, however, be substantiated because: (1) restoration of immunological capacity was evident in hosts bearing, for 10 to 14 days, thymus implants preirradiated with 500 r., whose regeneration pattern was identical with that of implants preirradiated with 2000 r.; (2) the presence for more than 2 weeks of implants preirradiated with 2000 r. failed to correct adequately the immunological defects of thymectomized mice (Miller *et al.*, 1966), even though such implants were fully regenerated after 3 weeks and filled with actively dividing host-derived cells. The failure of thymus tissue irradiated *in vitro* with 2000 r. to restore immunological responsiveness significantly may thus be related not to any delay in the appearance of thymus lymphocytes, but rather to an impairment of the capacity to induce differentiation or maturation of immunologically competent cells from non-competent precursors. This property of the thymus may be dependent on the integrity of its epithelial-reticular cells which may be responsible for the production of a humoral factor essential for the maturation of lymphoid cells to immunologically competent cells (Osoba and Miller, 1963, 1964; Miller, 1964). This function of the epithelial-reticular cells may not be resistant to doses of irradiation of 2000 r. and above.

There is a hint of a dissociation between thymus-dependent lymphopoiesis and thymus-dependent immunogenesis. Lymphopoiesis was as active in implants that had received 2000 r. as in those treated with 500 r.; yet only the latter implants significantly restored immunological capacity. Blood lymphocyte levels were within the normal range in most mice thymectomized at birth and bearing either normal thymus implants or implants irradiated *in vitro* with 500 or 2000 r. (Miller *et al.*, 1966). Furthermore, thymus tissue enclosed in Millipore diffusion chambers restored immunological capacity to neonatally thymectomized mice, yet many restored mice still showed deficiencies in the population of lymphocytes in blood and tissues (Osoba and Miller, 1963, 1964; Osoba, 1965).

The essential functions of the thymus may thus be (1) to provide lymphocytes by trapping lymphoid precursor cells from the circulation and causing their differentiation and proliferation to non-competent thymus lymphocytes, and (2) to elaborate a factor (competence-inducing factor) which directs the maturation or further differentiation of non-competent cells (possibly thymus-derived lymphocytes) to immunologically competent cells. The results obtained with thymus implants preirradiated *in vitro* and with thymus tissue enclosed in Millipore diffusion chambers suggest that the competence-inducing function of the thymus is dependent on the integrity of the epithelial-reticular cells.

SUMMARY

The thymus plays a central role in the development of immunological capacity. Thymectomy, when performed at an early age, interferes with the normal and complete development of the lymphoid system and impairs various immunological capacities: delayed sensitivity, homograft rejection and antibody formation to some, but not all, antigens. Thymectomy in adult life leads to no *immediate* significant impairment of immune capacity unless

the lymphoid system is damaged or destroyed by ionizing irradiation: under these conditions the anatomical and functional regeneration of this system is thymus-dependent. When animals thymectomized in adult life are challenged with a *new* antigen six or more months later, there is clear evidence of immunological deficiency.

The immune defects of thymectomized animals could be corrected by injecting suspensions of cells obtained from the lymph nodes and spleens of adult mice of the same inbred strain, or by grafting thymus tissue. By means of a chromosome-marker technique it was found that the dividing cell population in implants of normal thymus tissue was entirely replaced within 15 days by cells originating in the host. Furthermore, the majority of cells dividing in the lymph nodes and spleens were of host-type although in some cases thymus donor-type cells were found in increased proportion after antigenic stimulation. Thymus tissue initially depleted of lymphocytes by irradiation with 500 r. *in vitro* could also restore neonatally thymectomized mice, but in these cases no cells of thymus donor origin were found in the implant and lymphoid tissues whether the hosts were specifically challenged or not. By contrast, thymus tissue irradiated with 2000 r. failed to influence the immunological capacities of neonatally thymectomized mice even though the implants eventually became actively lymphopoietic.

The implications of these observations are discussed. It is concluded that the thymus plays an essential role in inducing the differentiation of immunologically competent cells from non-competent precursors, and that this function is dependent on the integrity of the thymus epithelial-reticular cells.

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DISCUSSION

Strauss: Dr. Miller, are the appearances of the grafts irradiated with 2000 r. and 500 r. similar in terms of reconstituted architecture?

Miller: In most thymus implants that had received irradiation with either 500 r. or 2000 r. both the architecture and the lymphopoietic activity appeared identical after a period of two to three weeks.

Metcalf: We have confirmed Dr. Miller's findings on the effect of simple thymectomy in several strains of adult mice using the haemagglutinin response to sheep red blood cells. Adult mice exhibit the normal capacity to form antibodies immediately after thymectomy but when we tested them at 9, 12 and 18 months after operation we found a progressively greater inability to form agglutinins (Metcalf, D. [1965]. *Nature, Lond.*, **208**, 1336). This suggests that, at least for the major part of the animal's life, the thymus plays a significant part in regulating immunological competence.

Miller: I meant to refer to Dr. Metcalf's findings on the delayed effect of thymectomy in adult mice. Dr. Roger Taylor, of the National

Institute for Medical Research in Mill Hill, has also obtained similar results using bovine serum albumen as the antigen (Taylor, R. B. [1965]. *Nature, Lond.*, **208**, 1334).

Auerbach: I have some experimental results which may be relevant to your interpretation of the different effects of irradiation of the thymus with 500 and 2000 r. We have found that when an embryonic thymus rudiment is isolated in tissue culture before it is lymphoid and irradiated with 500 or even 900 r. it will differentiate *in vitro* in exactly the same way as a non-irradiated thymus; that is, lymphopoiesis of the embryonic non-lymphoid cells is perfectly normal. But when we irradiated an embryonic thymus rudiment with 1200 r. no lymphopoiesis occurred. This suggests that embryonic systems which can undergo lymphoid differentiation are radiation-sensitive at about 1200 r. but not at lower doses. Dr. A. Globerson, working with adult thymus fragments, has shown that when a fragment is irradiated with 500 r. and explanted 24 hours later it regenerates *in vitro* in the absence of any outside stimulus, but when a higher dose of irradiation is given it does not regenerate. These two sets of results suggest to me that, in *in vitro* systems, there is a difference in the ability of the intrinsic pre-lymphoid cells of the thymus itself to undergo differentiation into lymphoid cells.

Miller: This certainly fits in with the results we have described. There is, however, one difference between your experiments and ours. In your *in vitro* system there can be no contribution from without: in ours, host cells migrate into the graft after about one week.

Auerbach: Yes, but the point is that the differentiation of the intrinsic lymphoid cells of the thymus seems to be inhibited by these higher doses of X-rays and not by the lower ones. The host lymphoid-cell population, of course, is independent of this and so I think that the parallel is probably a significant one.

Furth: Dr. Miller, I should like to ask you two questions: first, were the myeloid cells in your experiments fully mature?

Miller: There were some foci of what could be interpreted as immature cells—a few blast cells and some myelocytes—but there were many more fully mature myeloid cells.

Furth: Mature granulocytes are sometimes found in normal thymuses and thymus grafts and there has been some discussion about the role of

the thymus in myelopoiesis. Since the myeloid cells in your experiments were mature I think that they are not relevant to the problems we are now discussing.

My second question concerns your experiments with irradiated thymuses. It is interesting that irradiation with a dose of up to 2000 r. can cause a dissociation of two thymic functions. This suggests that the two—lymphopoiesis and the induction of immunological competence—are fundamentally different processes.

Miller: We have thought from these and other experiments that there may be a dissociation between thymus-dependent lymphopoiesis and thymus-dependent immunogenesis. I therefore agree with you that these may be two fundamentally different processes.

Kaplan: I think we should be cautious in drawing conclusions from the doses of X-rays that have been mentioned. Radiobiological experience indicates that responses to irradiation are really part of a continuum and that to establish the shape of a response curve one needs to know the responses to many different doses. I would be very doubtful if Dr. Miller's 500 and 2000 r. and Dr. Auerbach's 500 and 1200 r. give us an adequate notion of the form of this curve. We have studied the effect of still higher doses—in the range of 2000 to 5000 r.—and have found no regeneration of graft at these levels. So, at least in those grafts in which we have used 2000 r., our data do not support yours: we found neither regeneration nor involvement by lymphoid tumours in our grafts. I would like to see the response to a broader spectrum of doses before drawing conclusions from Dr. Miller's findings.

Miller: I agree that the response of thymus grafts to several different doses of irradiation must be tested. These experiments are time consuming and the only results available at the moment are the responses to 500 and 2000 r. The difference between your data for 2000 r. and ours may simply reflect strain differences.

Kaplan: We know from modern cellular radiobiology that the essential lesion produced by doses of X-rays in the range of 500–2000 r. is an impairment in the capacity for cell division. Most other functional capacities, such as the ability of cells to maintain protein and RNA synthesis, are reasonably well retained for many months after high doses of radiation provided the cells do not have to divide. Dr. Miller has

observed (this volume, p. 163), in thymectomized animals into which irradiated thymus tissue has been grafted, that there is a dissociation between the restoration of lymphopoiesis (which of course comes from the host) and the restoration of immunological capacity. If his observations are correct, then the cells in the thymus which are responsible for restoring the immunological capacity of the host must undergo cell division to accomplish this. Dr. Miller's results may, perhaps, be due to this known effect of radiation on cells.

Furth: Dr. Miller's observation reminds me of Puck's discovery (Puck, T. T., Marcus, P. I., and Cieciura, S. J. [1956]. *J. exp. Med.*, **103**. 273) that when cells are irradiated with about 5000 r. they function ("live") but do not proliferate. That is, they promote the replication of non-irradiated cells of the same type. To restore function in a thymectomized host a graft needs to imitate, *in vivo*, Puck's "feeder layer" of irradiated cells. Dr. Miller, are the cells still fully capable of reproduction, after irradiation with 2000 r?

Miller: Which cells do you mean? This is at least a two-cell system consisting of lymphocytes and reticulum cells.

Furth: The reticulum cells. I think that these are the source of the lymphopoietic factor. For use as a source of thymic factor *in vivo* they should be functional but unable to proliferate. The dose of X-irradiation is critical. I believe that the reticulum cells can still proliferate after exposure to 2000 r.

Miller: In cytological preparations we could not find any dividing cells in thymus grafts irradiated with 2000 r. until eleven to twelve days after grafting when lymphocytes appeared in the grafts. The dividing cells were then of the host type.

Burnet: Have any studies on karyotypes been made with appropriate material in these irradiated thymuses to see if, in fact, the donor-irradiated epithelial cells do proliferate?

Miller: In thymic implants irradiated with 500 r. we have sometimes, though rarely, seen dividing cells of donor type in the graft during the first week, and thus before the appearance of lymphoid cells. These dividing cells were presumably epithelial or reticular and their karyotype appeared to be normal. In thymic implants irradiated with 2000 r. no donor-type dividing cells were ever seen in cytological preparations.

Ford: In our studies of irradiation chimaeras we have sometimes found

restoration of host cells in the thymus after irradiation with 1000 rads and, in one outstanding instance, about 16 days after irradiation we found virtually complete repopulation of the thymus by a single clone: 70–80 per cent of the dividing cells were derived from one host cell that had survived this dose of X-rays.

Strauss: Dr. Clark, after Dr. Miller's presentation I would like to ask if you have studied acid mucopolysaccharide synthesis or secretion in irradiated thymus grafts?

Clark: No.

Woodruff: May I comment on the term "adult thymectomy"? I think it is misleading, especially since people tend to extrapolate from the mouse to other species in their thinking, to regard the mice that we are discussing as "adult". These animals are really teenage mice when you operate on them, although people tend to think of them as well-matured adults.

Metcalf: By the time mice are two months old ("young adults") they can already have had litters, so I would not regard them as teenagers unless they are very precocious! From the immunological point of view, a two-month-old mouse is a fully matured adult animal.

Woodruff: What would be the effect of thymectomy on mice of different ages? For example, if this work was repeated using six- to eight-month-old mice, would the results be reproducible? Has anybody ever done this?

Miller: We have thymectomized mice at the age of five months and followed this with 850 r. and syngeneic bone-marrow therapy. These mice failed to regenerate their lymphoid system functionally; foreign skin grafts were not rejected (Cross, A. M., Leuchars, E., and Miller, J.F.A.P. [1964]. *J. exp. Med.*, **119**, 837).

Auerbach: A. A. Axelrad and H. C. Van Der Gaag (1962. *J. Natn. Cancer Inst.*, **28**, 1065) and W. D. Ball (1963. *Exp. Cell Res.*, **31**, 82) have mapped out cell division in, and overall growth of, the thymus in mice. These workers found, depending on the strain of animal, that at about two months of age the slope for the increase in cell numbers in the thymus levels off and then becomes negative. These results suggest that at about two months thymus cell proliferation is at its highest.

Good: We have studied the development of immunological competence in spleen and lymph node populations with increasing age in

mice (Dalmasso, A. P., *et al.*, [1959]. *J. exp. Med.* **118**, 1089). The mice reach their maximum capacity for graft-versus-host reaction at about two to three months and this capacity does not vary after this for a long time. Differences in this process in different strains or individuals may be related to their experience with pathogens. When one examines pathogen-free pigs like those that Moore is studying in Texas (Moore, R. W., unpublished observations), one finds that the thymus in these old pigs is huge and like an immature thymus whereas, in conventionally reared animals, the gland shows considerable involution at the same age. Not only the development of the peripheral lymphoid tissue but also the involution of the central lymphoid tissue might depend on the animals' experience with pathogens and their antigens.

Gowans: The peripheral lymphoid tissue of thymectomized animals is presumably immunologically deficient either because it contains too few lymphocytes or because the lymphocytes that are present are defective. Dr. Miller, I assume that when you tested the ability of lymphoid cells from thymectomized animals to produce graft-versus-host reactions you tested equal numbers of cells from control and thymectomized donors.

Miller: When we injected 6×10^6 spleen cells from five-week-old C57BL neonatally thymectomized mice into newborn mice of a foreign strain there were no signs of graft-versus-host reactions in the recipients. By contrast, severe runt disease was produced by the same number of spleen cells from normal five-week-old C57BL mice.

Gowans: You measured the size of the inocula by the number of nucleated cells they contained?

Miller: Viable nucleated cells were counted: the counts, of course, included both lymphoid and myeloid cells. When we increased the number of spleen cells from thymectomized donors from 6×10^6 to 20×10^6, evidence of graft-versus-host activity was found in the neonatal recipients (Miller, J. F. A. P. [1963]. *In* La Tolérance Acquise et la Tolérance Naturelle à l'égard de Substances Antigéniques Définies, p. 47. Paris: CNRS).

Gowans: Do you think that thymectomized animals have defective lymphocytes which are morphologically the same as the competent cells of the normal animal?

Miller: I am certain that there are cells, morphologically similar to the small lymphocyte, which are not immunologically competent. Such cells have been described in the bone marrow and are thought to have stem-cell properties (Cudkowicz, G., Bennett, M., and Shearer, G. M. [1964]. *Science*, **144,** 866). You yourself, Professor Gowans, have pointed out that some of the small round cells originating in the bone marrow have morphological similarities to small lymphocytes, but have to be distinguished functionally from the small lymphocytes in lymph and lymphoid tissues (Volkman, A., and Gowans, J. L. [1965]. *Br. J. exp. Path.*, **46,** 62). The defects we have found in thymectomized mice may be explained on a quantitative basis: there may simply be less competent cells present in these mice than in normal animals.

Gowans: Do you really know if there is less competence per cell or are there just fewer competent cells?

Miller: Both. I think there are fewer competent cells in the thymectomized animal although we have not yet been able to prove this experimentally. I also think that there are cells, in both thymectomized and normal animals, which look like small lymphocytes (for example the small round cells in the bone marrow) but which are immunologically incompetent. Concerning the degree of competence per cell I do not know of any system which has been used to assay this.

Dameshek: Drs. Adner, Sherman and I made an immunological study of thymectomized adult patients with myasthenia gravis. These patients showed, rather consistently, a reduction in the blood lymphocyte count as well as reduced cellular (delayed) hypersensitivity, and an inconstant reduction in immunological capacity. (Adner, M. M., *et al.* [1964]. *New Engl. J. Med.*, **271,** 1327).

THE DEVELOPMENT OF THE CENTRAL AND PERIPHERAL LYMPHOID TISSUE: ONTOGENETIC AND PHYLOGENETIC CONSIDERATIONS

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In the past few years an extraordinary burst of research activity has been redefining the lymphoid system. At Minnesota, we observed the first patient with agammaglobulinaemia and thymoma and we did our first unsuccessful thymectomy experiments in rabbits between 1953 and 1955 (Good, 1954; Good and Varco, 1955; MacLean *et al.*, 1956). This was at about the time of Glick and his colleagues' first experiments on the immunological function of the bursa of Fabricius, a cloacal thymus-like mass of lymphoid tissue peculiar to birds (Glick, Chang and Jaap, 1956; Chang, Rheins and Winter, 1957; Glick, 1964). Stimulated by the extension of Glick's findings by Harold Wolfe and his students (Mueller, Wolfe and Meyer, 1960; Mueller *et al.*, 1962) we undertook neonatal thymectomy studies in our laboratories in Minnesota. We demonstrated a post-thymectomy depression of adaptive immunity first in rabbits (Archer and Pierce, 1961;

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Archer *et al.*, 1962) and then, in a more striking way, in inbred mice (Martinez *et al.*, 1962; Good *et al.*, 1962*a*). At about this time we initiated a new series of phylogenetic studies on immunity, concentrating on the cyclostomes and other primitive fishes (Good and Papermaster, 1961; Papermaster, Condie and Good, 1962). An important aspect of our studies was the group of immunologically deficient patients, particularly the agammaglobulinaemic-hypogammaglobulinaemic children and adults, who receive regular care in our hospital. We reviewed many aspects of this work comprehensively in 1962 (Good *et al.*, 1962*b*).

As the immunological and physiological consequences of neonatal or early thymectomy became evident in England (Miller, 1961, 1962) and in Sweden (Fichtelius, Laurell and Philipsson, 1961), as well as in our own laboratories, and as work on the bursa of Fabricius progressed, the first Conference on the Thymus took place—in 1962—and the proceedings were published in 1964 (Good and Gabrielsen, 1964).

During the last few years, as we have been working on the thymus-dependent and bursa-dependent lymphoid tissue of the chicken, and equivalent systems in mammals, reptiles, amphibians and fishes, we have often come back to two issues raised at that conference. The first was the apparent lack of relevance of thymectomy to the functioning of the immunoglobulin-producing system. Although neonatal thymectomy affected specific antibody production to some antigens (Good *et al.*, 1962*a*; Miller, 1962; Janković, Waksman and Arnason, 1962; Parrott and East, 1964) it seemed, in rabbits, rats and mice, to have little effect on plasma cells and γ-globulin (Janković, Waksman and Arnason, 1962; Azar, 1964; Parrott and East, 1964; Archer, 1964; Waksman, 1964). The second issue was the comment by Warner (1964) that a small number of his hormonally bursectomized chickens were agammaglobulinaemic and the observation of Warner and Szenberg (1964) that plasma cells were few in the bursa-less chicken. In the background of this problem was the postulate of

the Melbourne group (Szenberg and Warner, 1962; Warner and Szenberg, 1962; Warner, Szenberg and Burnet, 1962) on the dissociation of immunological functions in the chicken. These workers suggested that the bursa of Fabricius constituted the primordial tissue for the antibody-producing system, including delayed hypersensitivity, and the thymus governed the development of homograft immunity.

We had long been concerned, as had most immunologists, with the problem of accounting for two kinds of immunity—cellular and humoral. It seemed to us in 1962, at the time of the first Conference on the Thymus (*loc. cit.*) that the dissociation between the two might be attributable to different stages in the development of a single cell line. Several developments (*vide infra*) led us to accept the concept of dissociation if not the details of the model proposed by Warner and Szenberg, and to seek a clearer experimental demonstration of the roles of the thymus and bursa in the ontogeny of immunity. This was, we believe, achieved in the experiments of Cooper and his co-workers (Cooper, Peterson and Good, 1965, 1966; Cooper *et al.*, 1966; Peterson, Cooper and Good, 1965; Cooper, 1965).

Experiments by Janković and his associates (Janković and Išvaneski, 1963; Janković and Isaković, 1964) in the chicken supported the view that the chicken thymus, like the mammalian thymus, influences the ontogeny of cellular immunity of the delayed hypersensitivity type. The studies of our own group (Peterson *et al.*, 1963, 1964, 1966), in collaboration with Burmester and his associates at the United States Department of Agriculture Poultry Research Laboratory, East Lansing, Michigan, suggested that the major lymphoma of chickens, visceral lymphomatosis, is bursa-dependent and not thymus-dependent. Further support for the concept of dissociation was the finding of Ortega and Der (1964) that γG-agammaglobulinaemia could be produced in many chickens by surgical bursectomy followed by cautery in the newly hatched period. Our conclusions were also streng-

thened by the observation of Sutherland and co-workers (1965*a*), that neonatally thymectomized or thymectomized-appendicectomized rabbits often develop a positive Coombs' test and a variety of lymphoid-tissue abnormalities. These include depletion of small lymphocytes, deposit of amyloid, plasmacytosis, and reticulum cell proliferation, suggesting that the immunoglobulin-producing system is not only intact but hyperactive. At about this time deVries and co-workers (1964) reported the production of abnormal antibodies and histopathological lesions reminiscent of lupus erythematosus, in some strains of neonatally thymectomized mice.

Clinical findings also reinforced the concept of dissociation, particularly (*a*) the extreme γM- and γG-hypogammaglobulinaemia in the sex-linked recessive form (Bruton, 1952; Gitlin, Hitzig and Janeway, 1956; Scheidegger, 1956; Grabar, Burtin and Seligmann, 1958; Bridges and Good, 1960); (b) the definition of the immunological and morphological defects in the Swiss or lymphopenic form of agammaglobulinaemia (Glanzmann and Riniker, 1950; Tobler and Cottier, 1958; Hitzig *et al.*, 1958; Hitzig and Willi, 1961; Rosen, Gitlin and Janeway, 1962; Good *et al.*, 1963); and (c) the discovery of a variable immunological deficiency in ataxia telangiectasia (Thieffry *et al.*, 1961; Andrews, Kopack and Bruton, 1960; Gutmann and Lemli, 1963; Good *et al.*, 1963; Peterson, Blaw and Good, 1963; Peterson, Kelly and Good, 1964; Fireman, Boesman and Gitlin, 1964; Young, Austen and Moser, 1964).

To test the hypotheses that the bursa of Fabricius controls the development of immunoglobulin production in the chicken, that the thymus is probably largely responsible for the ontogeny of cellular immunity in the chicken as in mammals, and that some lymphoid development of the spleen and intestinal lymphoid aggregates is already incipient at hatching, Cooper and his associates added sublethal irradiation to thymectomy and bursectomy in the immediate post-hatching period (Cooper,

TABLE I

MODELS OF IMMUNOLOGICAL DEFICIENCY IN THE CHICKEN

	Procedures in immediate post-hatching period		
	Thymectomy-irradiation	*Bursectomy-irradiation*	*Bursectomy*
Morphological defect	Reduced small lymphocytes in blood and tissues; depleted white pulp; normal germinal centres and plasma cells	Lack of germinal centres and plasma cells; normal lymphocyte levels in blood and tissues	No consistent abnormality
Immunoglobulin production	Normal by immunoelectrophoresis	No γM- or γG-immunoglobulins by immunoelectrophoresis	A few birds normal by immunoelectrophoresis; many birds showed no detectable γG-, and increased γM-, immunoglobulins
Specific antibody production†	Significantly reduced	None	Greatly reduced
Delayed hypersensitivity†	Greatly reduced	Not tested	Normal
Graft-versus-host reactivity (Simonsen assay)	Greatly reduced	Normal	Normal
Homograft immunity	Greatly reduced	Significantly reduced	Normal‡

† Specific antibody to *Brucella abortus* and bovine serum albumen were measured in our studies; many other recorded experiments have defined the antibody deficit of chickens surgically bursectomized at hatching. The antigen in the delayed hypersensitivity studies was diphtheria toxoid.

‡ Data available only from skin homotransplants in this group; thymectomy-irradiation and bursectomy-irradiation groups were tested by a more sensitive wattle skin transplantation technique. Homograft immunity to dispersed lymphoid cells is deficient in birds subjected only to bursectomy.

Peterson and Good, 1965, 1966; Cooper *et al.*, 1966). The functional and morphological division of the two systems has been described in detail in these same papers and is summarized in Table I. The bursectomy-irradiation model was absolutely clear-cut: there were no germinal centres or plasma cells, γM- or γG-immuno-globulins by immunoelectrophoresis and no detectable antibody production to protein or bacterial antigens even after repeated stimulation. The number of small lymphocytes in the splenic white pulp, and the circulating lymphocyte levels, were normal as was the capacity for graft-versus-host reactions; there was definite, though modified, ability to reject skin homografts.

The immunological defect in thymectomized-irradiated chickens resembled that of neonatally thymectomized mice, rats and hamsters: there were impaired delayed hypersensitivity reactions and graft-versus-host capabilities; absent, or slow abnormal homograft rejection; and a reduced antibody response to both protein and bacterial antigens (Miller, 1961, 1962; Martinez *et al.*, 1962; Good *et al.*, 1962*a*; Arnason *et al.*, 1962; Janković, Waksman and Arnason, 1962; Roosa, Wilson and Defendi, 1963; Sherman, Adner and Dameshek, 1964; Humphrey, Parrott and East, 1964). As in mammals, the cell type most affected by thymectomy was the small lymphocyte which was reduced in the circulating blood and strikingly depleted in the splenic white pulp (Arnason *et al.*, 1962; Waksman, Arnason and Janković, 1962; Miller, 1962; Good *et al.*, 1962*a*; Defendi, Roosa and Koprowski, 1964; Cooper *et al.*, 1966).

The reclassification of clinical immunological deficiencies was already underway when these findings became available (Good, Finstad and Gabrielsen, 1964; Good, Peterson and Gabrielsen, 1964), the most astonishing parallel being that of the sex-linked recessive agammaglobulinaemic patient and the bursectomized-irradiated chicken (Peterson, Cooper and Good, 1965). There is a partial parallel with the thymectomy-irradiation model in ataxia telangiectasia (Peterson, Blaw and Good, 1963; Peterson,

Kelly and Good, 1964; Peterson, Cooper and Good, 1965), and another in some congenital lymphopenias associated with failure of thymic development but relatively intact immunoglobulin production (Nezelof *et al.*, 1964; Allibone, Goldie and Marmion, 1964). The Swiss form of agammaglobulinaemia seems to represent failure of development of both systems.

The experimental models of thymus-dependent and bursa-dependent lymphoid malignancies (McEndy, Boon and Furth, 1944; Kaplan, 1950; Law and Miller, 1950; Gross, 1963; Kunii and Furth, 1964; Peterson *et al.*, 1963, 1964, 1966) together with a range of clinical observations (Peterson *et al.*, 1965), led us to postulate that the lymphoid malignancies are, in their initial stages, diseases of one or other of the lymphoid-tissue components—the small lymphocyte component, typically thymus-dependent, or the germinal centre-plasma cell component, typically bursa-dependent (Peterson *et al.*, 1965). The initial involvement of one component is perhaps best seen in visceral lymphomatosis, in which the early morphological changes are proliferation and enlargement of the germinal follicles (Lucas *et al.*, 1954), followed by generalized overgrowth of malignant cells. There is good morphological evidence, in some of the classic investigations of human lymphoid malignancy, for an initial involvement of only one of the components (Ehrlich and Gerber, 1935).

The rest of this presentation will deal with three major unresolved issues: communication between the thymus-dependent and the bursa-dependent systems in the chicken and, by inference, in mammals and man; the bursa equivalent in mammals (drawing on both ontogenetic and phylogenetic studies); and the function and morphology of the lymphocyte. In conclusion we shall try to interpret the critical steps in phylogenetic development in terms of the pathological disturbances in the development of the human lymphoid system.

THE TWO-COMPONENT VIEW OF SPECIFIC ANTIBODY PRODUCTION

It is difficult to account for the depression of specific antibody production that follows neonatal thymectomy and thymectomy-irradiation, except by postulating that thymus-dependent cells, presumably small lymphocytes, furnish instructions or information to the immunoglobulin-producing cells. Two-cell or two-component theories of antibody formation have been recurrent during the last ten years or so, particularly following two specific observations. The first of these was the immunohistochemical confirmation that the plasma cell is the major production unit for specific antibodies (Coons, Leduc and Connolly, 1955; Leduc, Coons and Connolly, 1955), and the second, the definition by Dixon and associates (Dixon, Talmage and Bukantz, 1951; Dixon, Talmage and Maurer, 1952) of the radiosensitive and radioresistant phases of antibody production (see also Taliaferro, Taliaferro and Janssen, 1952; Raffel, 1956; Good, 1957). Communication between cells on the basis of transfer of DNA or RNA has been repeatedly postulated to explain such relationships. Several specific proposals of the mode of transfer of antibody specificity to immunoglobulins have recently been made. Burnet (1964) proposed the transfer of specific reactive sites in the form of short polypeptide chains, while Osgood (1964) suggested "liquid-crystals" as the vehicle for transfer of specificity to the immunoglobulin-producing cell; Smithies (1965) envisaged an "antibody virus" as the medium of communication. Non-antibody γ-globulin and non-specific stimulation of cells of the plasmacyte line seem to be necessary corollaries of these views.

The morphology of the two lymphoid systems is well seen in a rabbit lymph node, where the large lymphoid cells and typical germinal centre organization are surrounded in whole or part by a collar of small lymphocytes. The absence of the thymus-dependent component, the lymphocyte collars, is striking when adult rabbits are thymectomized and subjected to sublethal

irradiation (Peterson, Cooper and Good, 1965; Kellum *et al.*, 1965). The small lymphocyte collars are restored in control irradiated animals but they are lacking in thymectomized-irradiated rabbits whose lymph nodes, in section, show the germinal centres standing out sharply against the greatly reduced cellularity of the surrounding tissue. In the chicken spleen the germinal centres are also uniformly near to the small-lymphocyte, white-pulp type of lymphoid tissue, although the collar arrangement is generally lacking.

It has been less easy to restore immunological competence to bursectomized chickens than to thymectomized mice. Partial success has recently been reported with bursal homografts (Isaković and Janković, 1964) and with bursal tissue in Millipore chambers (Janković and Leskowitz, 1965; St. Pierre and Ackerman, 1965). In our own work with visceral lymphomatosis in an inbred line of chickens, we have obtained data suggesting a partial restoration of susceptibility to lymphoid malignancy after bursal grafting (Peterson *et al.*, 1966). Our findings are difficult to interpret because the line is not genetically homogeneous and most of the grafts are probably subject to homograft rejection. Bursal tissue in Millipore chambers did not seem to influence the protection against visceral lymphomatosis afforded by early bursectomy.

Cooper, Schwartz and Good (1966) have recently found that if autologous bursal cells are injected intraperitoneally into bursectomized-irradiated chickens, the birds develop germinal centres, plasma cells and circulating γ-globulins in normal or near-normal amounts, but remain incapable of significant antibody production to *Brucella abortus* or bovine serum albumen. Two possible explanations for these extraordinary findings are that a hormonal influence on immunological competence cannot be exercised by dispersed cells as opposed to intact tissue in Millipore chambers, or that the morphological restoration of the bursa-dependent system does not necessarily re-establish com-

munication between the thymic and bursal systems. We can, therefore, add another type of immunological deficiency to deficiencies involving partial or total absence of the thymus-dependent or bursa-dependent systems or both: this is a deficiency of communication between the systems, presumably in the receiving cells. These findings contrast with cell restoration experiments in thymectomized mice in which antibody-producing capacities have been restored by large inocula of thymus cells (Taylor, 1963).

Detailed studies of this and other restoration models in chickens are needed and offer the prospect of unusual insights into the mechanism of the specific antibody response.

THE MAMMALIAN IMMUNOGLOBULIN-PRODUCING SYSTEM

The bursa of Fabricius is peculiar to birds. The primordial tissue for the mammalian immunoglobulin-producing system is presumed to be another gut-associated glandular-lymphoid tissue, lympho-epithelial in type, relatively large in the embryo and neonate, and involuting at about the time of sexual maturity. We would expect it to be like the bursa structurally, with a follicular organization whose morphological appearance resembles that of the peripheral germinal centres. In the clinical disease offering the closest parallel to the defect in bursectomized-irradiated chicken, sex-linked recessive agammaglobulinaemia, such a structure should be undeveloped.

Several lymphoid structures meet some of these criteria: parts of the rabbit appendix and sacculus rotundus are structurally very similar to the bursa (Archer, Sutherland and Good, 1963, 1964; Peterson, Cooper and Good, 1965). This similarity was one of the bases for our first experiments involving neonatal appendicectomy with and without thymectomy (Archer, Sutherland and Good, 1963, 1964; Sutherland, Archer and Good, 1964). All the evidence so far suggests that the appendix in the rabbit

shares some aspects of thymic function, at least in the postnatal period (Sutherland, Archer and Good, 1964; Archer, Sutherland and Good, 1964); as yet there is no evidence that the immunoglobulin-producing system is affected by appendicectomy in the rabbit, although the development of the capacity to form specific antibody is depressed. Removal of the sacculus rotundus in the neonatal rabbit is difficult technically, and we have not yet had an adequate group of survivors for detailed study. The data that we do have do not support the notion that the sacculus is a functional equivalent of the bursa of Fabricius (Sutherland *et al.*, 1965*b*). Data on the immunological and haematological recovery of neonatally thymectomized-appendicectomized rabbits suggest that there is another collection of central lymphoid tissue of thymic type in the rabbit, and it seems probable to us that the sacculus is that tissue.

The leading candidates for the role of a bursa-equivalent in mammals are the pharyngeal and palatine tonsils. The virtual absence of these structures has been a major diagnostic criterion for the sex-linked form of agammaglobulinaemia (Margulis *et al.*, 1957) and morphologically they closely resemble the bursa (Peterson, Cooper and Good, 1965). The ontogenetic development of the pharyngeal and palatine tonsils roughly parallels that of the bursa and their phylogenetic development also suggests that the bursa represented a divergence from the main line of tonsillar development which occurred among the transitional species to the reptile-like birds (Good *et al.*, 1966). We are currently testing the hypothesis that the tonsils are the bursa-equivalent in mammals but we have no definitive data about this yet.

At the beginning of this century the thymus was studied extensively at all phylogenetic levels, and a wealth of morphological data about it is available. Less attention has been given to the phylogeny of the other lymphoid tissues, particularly in the cyclostomes and other primitive fishes. We have attempted to obtain a functional assessment of the immunological system in as

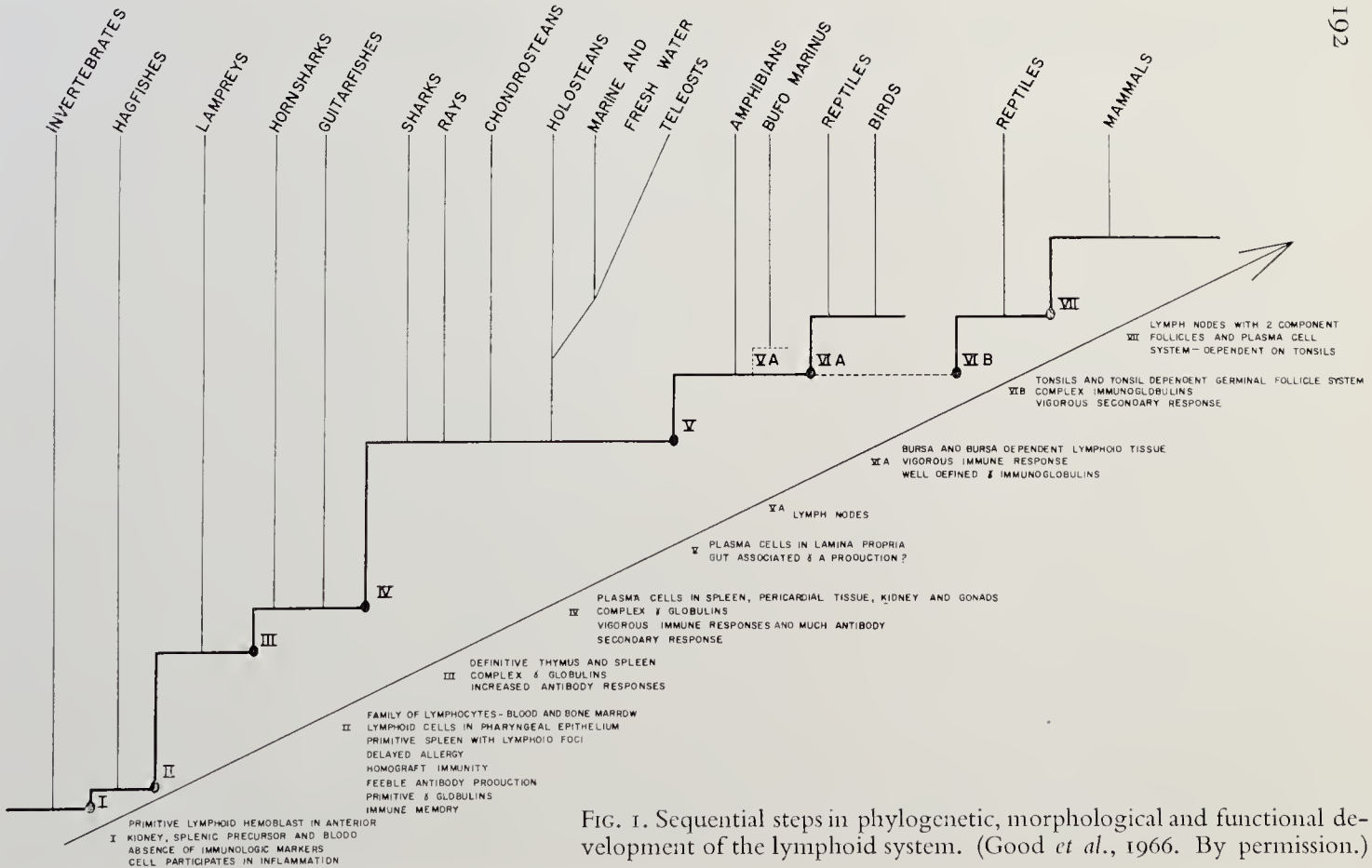

FIG. 1. Sequential steps in phylogenetic, morphological and functional development of the lymphoid system. (Good *et al.*, 1966. By permission.)

many of the lowest fishes as possible, correlated with the major advances in the phylogenesis of the lymphoid tissues and the lymphoid cell systems (Papermaster, Condie and Good, 1962; Papermaster *et al.*, 1964; Finstad, Papermaster and Good, 1964; Finstad and Good, 1964, 1966; Good *et al.*, 1966). Fig. 1 illustrates our current view of the major phylogenetic transitions, and is an attempted synthesis of what is known and a projection of studies to be undertaken. Three steps are of particular relevance when the ontogeny and phylogeny of immunity are considered together: the transition to the thymus and thymus-dependent type of development, the transition to a true immunoglobulin-producing system, and the transition to bursal and possibly tonsillar control of the immunoglobulin-producing system. The other steps presented in the figure are considered in detail elsewhere (Good *et al.*, 1966).

The hagfishes are generally considered the lowest existing vertebrate forms. We have studied extensively the California hagfish, *Eptatretus stoutii*, and, fairly extensively, the Atlantic hagfish, *Myxine glutinosa*. Neither of these has detectable adaptive immunity as we know it: they lack immunoglobulins, they do not form detectable antibody despite intense long-term antigenic stimulation and they do not develop delayed hypersensitivity (Papermaster, Condie and Good, 1962; Papermaster *et al.*, 1964; Finstad and Fichtelius, 1965; Finstad, Fichtelius and Good, 1965). The capacity for rejection of skin homografts of these two species of hagfish has not been well studied because the grafts tend to pull away from the graft site without healing. In the few instances in which some union has been achieved there has been no evidence of rejection. When small hepatic homografts and autografts were implanted, the cellular response was the same regardless of the source of the grafts. It is possible, however, to induce a type of inflammatory response in the hagfish, in which some of the infiltrating cells are lymphocytic in appearance; necrotizing responses do not occur (Finstad, Fichtelius and Good,

1965). We believe that the lymphocyte-like cell of the hagfish, called by Jordan and Speidel (1930) a lymphoid haemoblast, is a precursor of the red blood cell, granulocyte and thrombocyte, but that it has at least one of the attributes of lymphoid cells in higher forms, that is, a role in inflammation.

The lampreys represent an advance over the hagfish in several ways. It was of great interest to us to find that the lamprey (*Petromyzon marinus*) has a thymus-dependent type of cell system and immunological function, that is, it shows delayed hypersensitivity and homograft immunity (Finstad and Good, 1964). It does not have the thymic structure seen in the gill regions in fishes higher in the phylogenetic scale but it has small aggregates of lymphoid cells in the pharyngeal gutters from the second to the fifth gill arches. These aggregates were described fifty years ago (Salkind, 1915) as a lympho-epithelial thymus. In contrast to the hagfish, the lamprey has intense, and often fatal, necrotizing reactions to injections of Freund's adjuvant. It has lymphoid aggregates in the spleen, and large and medium, as well as small, lymphoid cells. It responds with feeble antibody production to *Brucella abortus* but fails to respond to serum protein antigens, haemocyanin or bacteriophage (Finstad and Good, 1964). Immunoelectrophoresis reveals at least two proteins of gamma mobility in a relatively simple pattern (Finstad and Good, 1964; Pollara, Finstad and Good, 1966*a*). We have found no cells resembling plasma cells or plasma-cell precursors on light microscopy. Thus, almost all the concomitants of the bursal-type of development are lacking, despite the presence of small amounts of two globulins of gamma mobility and small amounts of antibody. Attempts to identify the two globulins have been unsuccessful (Ashbach *et al.*, 1964; Pollara, Finstad and Good, 1966*a*). One of the globulins seems to have fast gamma mobility and may be contained in the faster migrating fraction on immunoelectrophoresis. Very recent, and as yet incomplete, data indicate that in the secondary immune responses more vigorous produc-

tion of antibody may be observed in the lamprey and that this antibody may have the characteristics, on density gradient and ultracentrifugal analysis, of γG-immunoglobulin (Pollara, Finstad and Good, 1966*b*). Analysing the problem of a partial bursal-type of system in the lamprey may help to elucidate the derivation of the two lymphoid systems and their separate or co-ordinate function. Several experimental approaches to this problem are currently being made.

The chondrosteans (we studied the paddlefish, *Polyodon spathula*) are of enormous interest because they have plasma cells and plasma-cell precursors with classical Marschalko characteristics on light microscopy (Good *et al.*, 1966) and typical ergastoplasm and endoplasmic reticulum under the electron microscope (Clawson, Finstad and Good, 1965). The paddlefish show vigorous immune responses including a surprising range and intensity of antibody response (Good and Finstad, 1964; Finstad and Good, 1966) and a curious heavy-weight antibody globulin which is extraordinarily resistant to cleavage or reduction by 2-mercaptoethanol (Fish, Pollara and Good, 1966). They also have a well differentiated thymus, splenic white pulp, and lymphoid tissue in the gonads and anterior kidney and along the gut tract (Good *et al.*, 1966). A true germinal-centre type of lymphoid organization is not present in the paddlefish but there is striking cloning of plasma cells in the spleen in the secondary antibody response. Germinal centre organization is not seen in any of the fishes despite vigorous antibody responses in some of them. Since this type of follicular arrangement is the hallmark of the bursa-tonsil tissue, this may be the reason for our failure to identify any bursa-like structure in the paddlefish. Fluorescent antibody studies are needed in the chondrosteans and in the lower fishes to help to identify cell types containing specific antibody and presumably producing it.

The last transition—more conjectural than the other two but perhaps more relevant to the unresolved problems of the two lymphoid-tissue components and their interrelationships—is

the transition to lympho-epithelial tonsillar development in mammals and bursal development in the early kinds of birds. The bursal type of development and its counterpart in monotremes, marsupials and mammals probably had their origin in extinct reptilian ancestors. Since plasma cells clearly preceded the appearance of recognizable tonsils or bursae phylogenetically, additional studies of lymphoid tissue aggregates are needed in reptiles, and in the fishes showing a vigorous secondary antibody response such as the paddlefish. One of the characteristics of both thymic and bursal tissues in higher forms is a relative aloofness from active immunological processes. This has been well documented for the mammalian thymus (Bjorneboe, Gormsen and Lunquist, 1947; Fagraeus, 1948) and apparently is also true for the bursa of Fabricius (Dent and Good, 1965). A variety of techniques are available for assessing the capacity of various cell populations for antibody production and might well be employed in the search for bursa-equivalents in lower animals where clear-cut morphological criteria are not available. Studies of the pharyngeal lymphoid tissues of the monotremes might also be significant in the search for the steps involved in the development of the lympho-epithelial tonsil.

One of the paradoxes of the phylogenetic studies has been the extraordinary vigour of cellular responses in the lamprey, a species relatively incapable of specific antibody production (Finstad, 1964; Finstad and Good, 1964). When antibody responses to a battery of antigens at different phylogenetic levels are measured, the trend is toward a broader range of sensitivity and greater vigour of response as we go up the scale, but there are many exceptions (Finstad and Good, 1966). Homograft immunity, on the other hand, is quite consistently efficient in all the fishes, except the hagfishes, at suitable water temperatures. This suggests that the failure of response is less one of recognition of antigenicity than of specificity of antibody response, perhaps because of a limited repertory of proteins or polypeptides, or more

limited communication between the two lymphoid cell systems in the lower species.

THE LYMPHOCYTE

Since our earliest work on the hagfish we have been preoccupied with the lymphoid haemoblasts, the lymphocyte-like cells which seem to be stem cells and, in a very limited sense, functional lymphocytes, but functional only in inflammation. Many of the lymphocytes of higher animals may be of this type, particularly the lymphocytes of the bone marrow which are of such limited capability in graft-versus-host reactions (Billingham and Silvers, 1964) and do not restore immunological function in thymectomized irradiated mice (Miller, Doak and Cross, 1963). The basic barrier to true immunological activity in the hagfish may not be lack of lymphocyte precursors but of thymic hormone or perhaps thymic cell-to-cell influence. Ford and Micklem (1963) found that there is selective migration of bone-marrow cells to the thymus of lethally irradiated mice, while other types of lymphoid cells are generally excluded.

Two recent findings, one clinical and the other experimental, are relevant to this problem. Hitzig (1964) and his colleagues in Switzerland (one of the investigative groups which defined the lymphopenic (Swiss) form of agammaglobulinaemia) attempted to reconstitute immunological function in an infant with this syndrome using foetal liver. This produced a considerable increase in the circulating and tissue lymphocytes but no change in the immunological status of the child. This suggests that the cells making up the reconstituted lymphocyte population were mostly primitive lymphoid haemoblasts which were unable to exercise their immunological function because of the absence of thymic activity in these infants. Similar results have been obtained by Rosen (1965) and his associates. The thymus in the Swiss type of agammaglobulinaemia is usually a tiny piece of tissue, devoid

of lymphoid cells and Hassall's corpuscles, and probably incapable of significant function.

The second relevant finding is the observation by Thomas *et al.* (1965) that the bone marrow contains a population of lymphocyte-like cells with the capacity to proliferate and differentiate in erythroid, myeloid and possibly lymphoid directions. These investigators compared the recovery pattern of dogs given near-lethal doses of irradiation or nitrogen mustard: the mustard-treated animals recovered faster. They also found that bone marrow from animals treated with nitrogen mustard could hasten the haematological recovery of X-irradiated dogs, whereas

TABLE II

MAMMALIAN LYMPHOCYTE MARKERS

1. Classic morphology and staining
2. Electron microscopic characteristics
3. Susceptibility to adrenal steroids
4. Susceptibility to irradiation
5. Susceptibility to nitrogen mustard
6. Susceptibility to a dumping action of *Haemophilus pertussis*
7. Capacity to be stimulated to blast cell differentiation by phytohaemagglutinin
8. Ability to participate in acute and chronic inflammatory reactions
9. Ability to function in immune responses

the bone-marrow of X-irradiated animals could not. This suggests that a stem-cell population was spared in the nitrogen-mustard treated dogs, and this conclusion is supported by morphological and radioautographic analyses of serial bone-marrow samples from the dogs which had received nitrogen mustard. Both *in vivo* and *in vitro* studies suggested that the stem cells are initially lymphocyte-like and that they develop as haemocytoblasts, and later as granulocytes, erythrocytes and other cells.

Our present investigations, again in the primitive fishes, are designed to define the phylogenetic development of lymphocytes and their responses in terms of nine parameters which we use as markers for mammalian lymphocytes (Table II). In these investi-

gations we are comparing various tissues: (a) the round cells of the white gland of the octopus; (b) the peripheral blood and haematopoietic tissue of the anterior kidney and gut of the hagfishes; (c) the primitive spleen, pharyngeal gutter, protovertebral arch and peripheral blood of the lamprey; and (d) the thymus, spleen, gut and peripheral blood of sharks, skates, rays, chondrosteans, holosteans, and marine and freshwater teleosts. Using the nine markers of lymphocytes (Table II), we hope to define the origins of the functional capacities and characteristics of lymphocytes as we know them in mammals.

CLINICAL IMMUNOLOGICAL DEFICIENCY

The broad range of immunological deficiency syndromes seen in clinical practice offers additional insights into the key steps in phylogenetic development outlined in Fig. 1. "Immunopathology recapitulates phylogeny", and failure to take sequentially any of these steps leaves a gap or blocks a sequence of development. Failure in ontogenetic development to make each transition, which is certainly under genetic control, has predictable consequences that in at least six of the seven phylogenetic steps can be associated with clinical disease in man. For example, failure to take step I (see Fig. 1) parallels the reticular dysgenesia of de Vaal and Seynhaeve (1959) in which infants, on the basis of an autosomal recessive trait, are unable to form lymphoid cells or granulocytes and live only a matter of days. Failure to take step II may be compared to the classic Swiss agammaglobulinaemia in which the thymus is rudimentary, non-lymphoid and located in an undescended position in the thyroid area, and in which there is no peripheral lymphoid development. The difference between this form of lymphopenia-agammaglobulinaemia and the thymic alymphoplasia of Rosen, Gitlin and Janeway (1962) seems to be analogous to the difference between steps II and III in the figure. In thymic alymphoplasia the thymus is present as a

definitive organ and has undergone descensus, but has not developed as a lymphoid organ. A block at step V might give the isolated inability to synthesize γA-immunoglobulin (Rockey *et al.*, 1964), and perhaps characteristics of the ataxia telangiectasia syndrome (Thieffry *et al.*, 1961; Peterson, Kelly and Good, 1964; Fireman, Boesman and Gitlin, 1964; Young, Austen and Moser, 1964) and other disorders.

Step VI represents the development of the bursal or bursa-equivalent system. At this step, at least in chickens, full control of germinal-centre and plasma-cell development seems to have centred in the bursa. Developmental failure here could be the basis for sex-linked recessive agammaglobulinaemia. Experimentally this same kind of disease has been achieved in the chicken by bursectomy and irradiation in the newly-hatched period (*vide supra*). In both the clinical syndrome and the laboratory model, the thymus system seems to remain intact.

Finally, a block in maturation of the follicular and plasma-cell system at a stage of partial differentiation (step VII) might give rise to the dysgammaglobulinaemia of Israël-Asselain, Burtin and Chebat (1960) and Rosen *et al.* (1961). Cooper *et al.* (1966), have recently produced this type of immunological defect experimentally in chickens. These animals are made deficient in γG-immunoglobulins by complete bursectomy at hatching, and their γM-immunoglobulins are regularly found to be increased. They remain seriously handicapped in their capacity for specific antibody response. Other chickens, bursectomized in the newly-hatched period, develop γG-immunoglobulins and show no increase of γM-immunoglobulins, perhaps because the lymphoid development in these birds is more advanced at hatching. They are, however, very deficient in antibody production.

Further detailed analysis of the phylogenetic development of the lymphoid system and its functions may reveal other parallels with human disease. In human pathology we should expect to

encounter an infinite variety of genetically determined diseases reflecting the trail of development of those physiological processes which had survival advantage in evolutionary development.

SUMMARY

The work of the last ten years on the thymus, the bursa of Fabricius and other central lymphoid tissues is the basis of a new view of the lymphoid system. This has drawn heavily on investigations of the ontogenesis and phylogenesis of adaptive immunological processes and their cellular and humoral correlates, and on studies of the experiments of nature observed as clinical immunological defects in clinical practice. Two models of immunological deficiency in the chicken, one thymus-based and the other bursa-based, have provided the basis for the re-evaluation of clinical and experimental immunological deficiency as well as clinical and experimental lymphoid malignancy, and have reorientated the direction of our research on the central lymphoid tissue in both ontogeny and phylogeny.

This work supports the concept that there is a primitive lymphocyte-like cell, the lymphoid haemoblast, which plays a general role in haematopoiesis but does not express immunological characteristics until it has undergone some degree of differentiation.

The phylogenetic development of the lymphoid system can be viewed as a series of steps which, when blocked in the ontogenetic development of man, may be expressed as different forms of immunological deficiency.

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DISCUSSION

Burnet: I am going to presume on the fact that we are meeting in Australia to interpolate a little material about some Australian animals —the monotremes and the marsupials; this seems particularly relevant after Professor Good's paper. I shall talk about the echidna or spiny ant-eater (*Tachyglossus aculeatus*), an egg-laying monotreme, and Professor Stanley will say something about the thymus in marsupials.

Dr. Erwin Diener and I have studied the lymphoid system of the echidna and we have found that the lymphatic system is of an extraordinarily primitive character. There are no lymph nodes in the ordinary sense but there are what look like congeries of fairly large vessels within which are isolated nodules. These seem to be single primary follicles and often have a pale centre in which proliferation occurs. The lymphoid system of the echidna also includes a thymus in the thorax (we have not found a cervical thymus so far) and this is a rather normal looking structure with Hassall's corpuscles, cortex and medulla. In the several small appendices associated with the bowel in these creatures there are epithelial-lymphoid structures which resemble the picture that Professor Good has described in the rabbit. E. Diener and E. Ealey (1965. *Nature*, **208**, 950) are studying the production of antibody in the echidna and their work should make a significant contribution to immunology since the echidna is by far the most primitive mammal available for study.

These preliminary findings suggest to me that investigation of the echidna may play an important role in correlating Professor Good's, and others', findings in the chicken and in mammals.

Stanley: Professor H. Waring, M. Yadav and I (unpublished observations) in the departments of Zoology and Microbiology at the University of Western Australia, have been carrying out preliminary immunological investigations on marsupial pouched young. I wish to report our early studies on one marsupial—the quokka (*Setonix brachyurus*) (Fig. 1). This marsupial is largely confined to Rottnest Island, ten miles off the Australian coast at Perth. The quokka is found in large numbers on this island and resembles a small wallaby, not more than two feet high when erect. Like some other Australian marsupials, but not all, the quokka has an external thymus situated bilaterally in the neck, as

well as the usual internal one. At birth the internal and external thymus glands are close together, separated only by connective tissue.

After a 28-day gestation period the embryo (now referred to as a joey) migrates to the pouch and becomes firmly attached to a teat. This process must represent the first contact of the joey with a numerous and varied microbial flora.

Fig. 1 (Stanley). The quokka (*Setonix brachyurus*).

Although the external and internal thymuses are similar at birth, differences in the mass and morphology of the two organs occur with development. At birth the joey weighs between 200 and 400 mg. Fig. 2 shows the relative masses of the external and internal thymuses with increasing age. Between 90 and 180 days the mass of the external thymus greatly exceeds that of the internal. At six days small lymphocytes become apparent in both external and internal glands and, at 19 days, the external thymus begins to show cortical and medullary regions, followed a few days later by the development of Hassall's corpuscles in the medulla and densely compact small lymphocytes in the cortex. At about 90 days the main difference between the external and internal thymuses is their mass and the number of lobulations. During the next 30 days the external thymus shows a great increase in the diameter of the lobulations. Between 120 and 125 days shivering is first noticed, the joey stands erect and underhair is apparent. At 180 days the joey can leave the pouch.

Experiments were performed to determine the antibody response of newborn quokkas to the inoculation of sheep red blood cells. Some of the quokkas were intact, whilst others had the external thymus excised at various times after birth. The results of tests for the presence of antibody are summarized in Table I. From our findings we

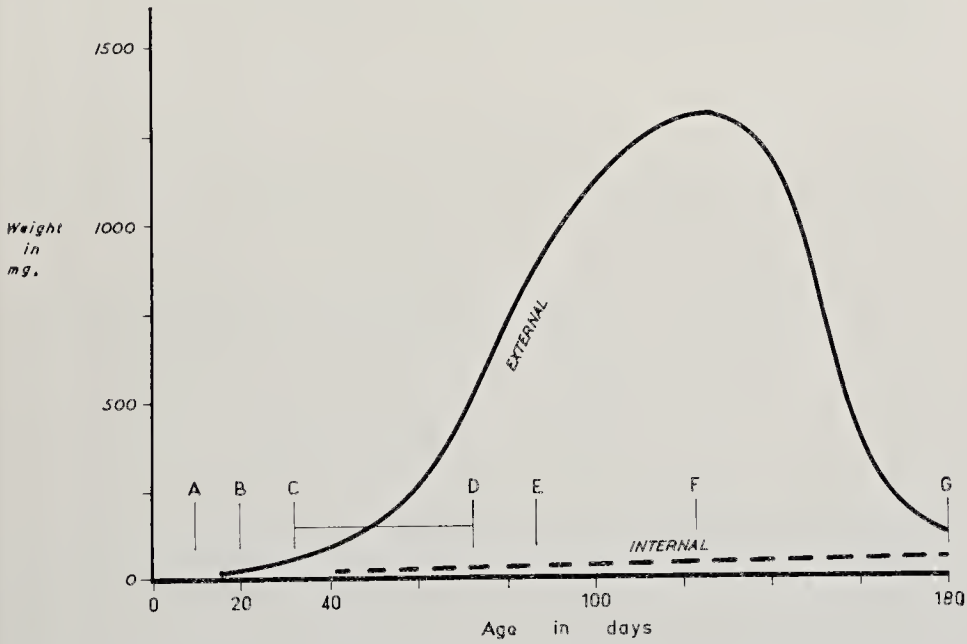

FIG. 2 (Stanley). Weight of internal and external thymus glands of quokka (*Setonix brachyurus*) during pouched life. A. Small lymphocytes apparent (both glands). B. Cortex and medulla commence to differentiate (external). C and D. Densely compact small lymphocytes in cortex (both glands). Hassall's corpuscles located. E. Large increase in diameter of lobules of external thymus. F. Stands erect, underhair first apparent. G. Joey leaves pouch.

have concluded that early inoculation of sheep red blood cells may produce partial tolerance in intact animals. External thymectomy before 24 days of age has a significant inhibitory effect on antibody production against sheep red blood cells and significantly reduces the lymphyocyte count. We have not been successful in our efforts to perform internal thymectomies on a limited number of quokkas before the age of 20 days. External thymectomy is relatively easy, but it must be

remembered that all this work, including the inoculation of red blood cells and the collection of blood is done while the very small joey is in the pouch attached to the teat.

Antibody was estimated by a micro-capillary technique which is effective with as little as 0·005 ml. of serum. Histological studies are being carried out to locate other lymphoid tissue but, to date, a classical bursa of Fabricius and tonsils have not been found.

TABLE I (Stanley).

ANTIBODY RESPONSE OF INTACT AND EXTERNALLY THYMECTOMIZED QUOKKAS TO THE INOCULATION OF SHEEP RED BLOOD CELLS

	Age thymectomized (days)	*Age inoculated (days)*	*Ratio of number with antibody to number tested*	*Differential leucocyte count (per cent)*		
				lymphocytes	*polymorphs*	*monocytes*
Intact	–	<10	0/3			
	–	10–12	2/9	45·0	39·0	16·0
	–	13–90	10/12			
	–	>90	4/4			
External thymectomy	6	5–8	0/3	26·6	59·8	13·6
	<24	16–38	0/11			
	>25	32–58	7/9			

It is possible that the external thymus has evolved in the quokka to help the joey, after the 28-day gestation period, to combat the microbial flora of the pouch. A study of the relationship between the external and the internal thymus glands in the quokka, and in other marsupials with no superficial thymus, may throw light on the evolution of the immunological mechanisms involved.

Good: This is an extremely important segment of phylogenetic inquiry. During the past five years we have been working to understand the phylogenetic development of the lymphoid system and immune responses. It is here in the no man's land between the reptiles, the birds and the mammals that a very critical step, probably related to the ultimate relationship of the follicular and plasma-cell systems,

probably took place. Phylogenetically, plasma cells appeared in organisms reflected in the modern elasmobranchs, chondrosteans, and holosteans perhaps as much as two hundred million years before the quokkas and monotremes, and even the mammals, appeared on the earth; and yet the follicular structure which you see in the echidna, as well as in the quokka and other marsupials, and the distribution of the lymphoid organs of these animals are certainly comparable to the distribution of cervical, axillary and thoracic lymph nodes in the mammals. In the dissections of echidna that I have carried out, these aggregates of monofollicular lymphoid tissue are very scanty in the posterior part of the body, but are appropriately located along the lymphatics especially in the cervical, axillary and thoracic regions. I think that they are primitive lymph nodes—the most primitive true two-component lymph nodes that have been described. This is especially important because reptiles and most birds lack lymph nodes. The juxtajugular bodies of the Amphibia are not, in my view, true lymph nodes even though they are probably an important step in their development.

Miller: Do reptiles have bursae?

Good: None of the modern reptiles that I know of have true bursae. The first appearance of lymph node tissue was probably in an ancestral form of the Amphibia because in *Bufo marinus* and *Rana pipiens* there are very simple lymphoid agglomerates along the blood vessels in the jugular region. In the reptiles three or four deviant lines evolved, separating out in the ancient cotylosaurs. Reptiles have been described with lympho-epithelial tissue in association with the posterior intestine. None of the modern reptiles reflect the reptilian line leading to the birds; the line of reptiles that *is* reflected today in the birds developed lympho-epithelial tissue of the bursa type. The reptilian ancestors of the mammals and monotremes are also extinct and not available for study. However, in most of the reptiles that I have studied I have found a primitive lympho-epithelial tonsil and I think that this represents the earliest occurrence of a true tonsillar structure in which epithelial and lymphoid tissues intermingle as in the mammals. These structures probably persisted into the mammals where there has been organization into two complementary sets of lymphoid tissue, as the development of the "lymph nodes" in the echidna suggests. In these primitive lymph nodes there is clearly a peripheral zone of small lymphocytes and

a central zone of larger cells just as in mammalian lymph nodes, but in the echidna each node is one follicle. The echidna also has abundant plasma cells.

Furth: In our experience of the avian leukoses the thymus is always normal and not even the site of metastases. We wanted to find out if there is an early stage when these neoplasms are still dependent on a hormone or some other factor. Bursectomy is said to inhibit lymphoma, up to about five months, and it is possible that if the bursa is removed earlier, with or without thymectomy, lymphoma might be transplantable only to animals that have been operated on. I think it would be useful to do some experiments to discover if there is such a condition as a hormone-dependent lymphoma and the bird is the ideal experimental model for such experiments.

Good: I agree. Drs. Peterson, Cooper and I have done some similar work to yours in collaboration with B. R. Burmester at the Regional Poultry Laboratories at East Lansing, Michigan. (Peterson, R. D. A., *et al.* [1964]. *J. Natn. Cancer Inst.*, **32**, 1343; [1965]. *J. Natn. Cancer Inst.*, [1966]. In press). The bursa was removed at hatching from fowls that had been inoculated with the infecting virus, RPL 12, in the embryonic period when the thymus was undifferentiated. Using this procedure we were still able to prevent visceral lymphomatosis, which suggests that the thymus is not involved in this. Even in these previously infected animals bursectomy arrests the disease although I do not think that it actually prevents lymphomatosis once it is established. This neoplasm of the lymphoid system is apparently bursa-dependent for as long as one can see and identify the bursa as an organ. Visceral lymphomatosis in fowls may really be a hormone-dependent lymphoid malignancy. It may indeed be a suitable system in which to study stages in the differentiation of the malignant condition and the role of hormones in the development of the malignant process.

Furth: Before full hormone dependence there is, in other tumour systems, a hormone-responsive autonomous phase. In your system, a lymphoma that is hormone dependent would only be transplantable when the bursa is absent. When the tumour is partially hormone responsive it would grow in normal chickens to some extent, but would progress much more rapidly in bursectomized (and perhaps also thymectomized) hosts.

Good: We are at the moment trying to transplant the visceral lymphomatosis tumour, at various stages in its development, into bursectomized chickens but we have no results from this yet. An interesting finding is that the other malignancies, osteopetrosis and erythroblastosis, induced by this same virus (RPL 12) are not prevented by bursectomy; indeed, if anything they are enhanced by it.

THE FUNCTION OF THE THYMUS IN TUMOUR PRODUCTION BY POLYOMA VIRUS†

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There is an impairment of immunological competence in most strains of mice subjected to removal of the thymus in the neonatal period. The immunological deficits include defects in humoral antibody formation, the delayed type of hypersensitivity and homograft rejection. The immunological deficiency is not generalized, since the capacity to respond to some antigens is unaffected (Humphrey, Parrott and East, 1964; Fahey, Barth and Law, 1965). The ability to reject allogeneic grafts of normal or neoplastic tissue, however, appears to be generally influenced, especially when antigenic differences between recipient and donor are slight. Usually, depending on the strain, lymphoid tissues are underdeveloped and there is a concomitant deficiency of small lymphocytes throughout the body.

The animal with a deficient immune mechanism is useful for studies of host resistance to spontaneous and experimentally induced neoplasms, particularly to those reported to be antigenic in the syngeneic or autochthonous host and which might be expected to initiate a homograft type of reaction. The frequency and progression of neoplasms induced by certain chemical carcinogens and by polyoma virus are indeed reported to be influenced by thymectomy (Miller, Ting and Law, 1964; Law, 1965*a*, *b*; Mori, Nomoto and Takeya, 1964; Vandeputte *et al.*, 1963; Defendi and Roosa, 1964; Miller, Grant and Roe, 1963; Grant and Miller, 1965.)

† Dr. Law was unable, at the last moment, to be present and this paper had to be sent after the meeting; there is, therefore, no discussion of it. Eds.

Polyoma virus inoculated into newborn mice multiplies to a high titre in many organs, produces an antiviral antibody response and, after a fairly short latent period, induces neoplastic growths particularly in the salivary glands but in other tissues as well. C57BL and A strains of mice, however, are resistant to the oncogenic effects of the virus introduced at birth and behave as adult animals of the susceptible strains in spite of the fact that polyoma virus again multiplies and antiviral antibodies are produced. That resistance to oncogenesis by the polyoma virus is related to defence mechanisms operating at the level of the organism is shown by the ability of the virus to transform organ explants of salivary tissue from C57BL and A strains of mice, into neoplastic growths (Dawe, 1965).

The present paper reports the results of studies designed to gain some knowledge of the relation of immunological competence to the induction of neoplasms by several methods, but particularly by polyoma virus.

EXPERIMENTAL DATA

INHERITABILITY OF RESISTANCE

The genetic basis of resistance in C57BL/Ka mice to tumour induction by polyoma virus was investigated by testing the ability to resist virus in the segregating populations derived from crosses of C57BL with a highly susceptible strain, C3Hf/BiLw. All mice delivered of polyoma, haemagglutination inhibiting (HI)-negative mothers were inoculated at birth with a thymotrophic strain of virus, P-388 D_1 (Dawe, Law and Dunn, 1959). Thymic epithelial neoplasms and pleomorphic neoplasms of the salivary glands were the common morphological forms observed in this group of mice. Fig. 1 shows the cumulative frequencies observed in these resistant and susceptible inbred strains, that is, the F_1 mice (including reciprocal crosses), and backcross mice to the resistant ($F_1 \times$ C57BL) and to the susceptible ($F_1 \times$ C3H)

parents. All C3H mice developed neoplasms by six months; none out of 52 C57BL mice developed tumours throughout a 14-month observation period. The results from the F_1 and back-cross generations strongly suggest that a single gene or only a few

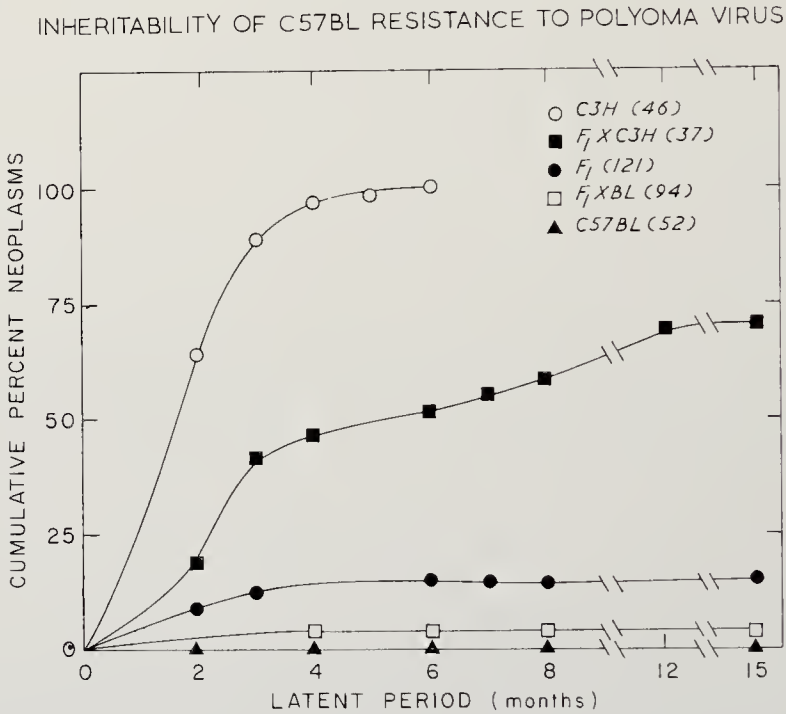


FIG. 1. Cumulative incidences of polyoma-type neoplasms (primary parotid gland tumours) in resistant C57BL/Ka and susceptible C3Hf/Bi mice, their F_1 hybrids—both (C3H × C57BL) and (C57BL × C3H)—and backcrosses of F_1 to resistant and sensitive parents. Numbers in parenthesis = total numbers of mice observed during 15 months. P-388 D_1 polyoma virus injected at birth.

genes are the major determinant of resistance to polyoma-induced tumour formation. In the absence of definitive progeny tests an unequivocal judgment cannot be made. Single genes are known to determine host susceptibility or resistance to certain other viruses (Sabin, 1952; Bang and Warwick, 1960; Lilly, Boyse and Old, 1964). Exactly how the determinant gene or

genes function is not known; however, C57BL strain mice do become immunologically competent soon after birth, earlier than most other inbred strains, and are known to be relatively resistant to the induction of tolerance (Möller, 1961; Billingham and Brent, 1959).

Recently Rubin (1965) has reported that susceptibility to pseudotypes of the Rous sarcoma virus (RSV) is determined by a single pair of autosomal genes and that tumour induction is determined by the responsiveness of the target cells. Susceptibility and resistance are therefore expressed *in vitro* as well as in the live chicken, in contrast to the response of C57BL mice to polyoma virus.

There is good evidence that neoplasms arising after infection with polyoma virus have specific antigenicity (Sjögren, Hellström and Klein, 1961; Habel, 1962; Defendi and Roosa, 1964; Ting and Law, 1965). This "new" antigen is apparently similar in mice from tumour to tumour and from strain of virus to strain of virus, although preliminary evidence from two laboratories now suggests that qualitatively different antigens are induced by the LID-I and the SE 3049 strains of polyoma virus (Hare, 1964; Ting, 1964). Adult mice, after being given an immunizing dose of virus, become resistant to the transplantation of syngeneic polyoma-type tumours but not to other neoplasms. This phenomenon is best illustrated in Table I: when groups 1 and 3 are compared it is seen that mice given an inapparent infection with the LID-I strain of polyoma virus three weeks before challenge with polyoma-induced tumour 4198, resist challenge with 2×10^5 cells whereas 25 out of 26 non-infected mice grew the neoplasm progressively and died in three to four weeks.

The refractoriness of virus-immune adult mice to transplantation of syngeneic polyoma-induced tumours may be explained on the basis of new virus-specific cellular antigens in the transformed cells. Habel (1962) and Sjögren, Hellström and Klein (1961) first developed this concept. Virus inoculation of adult

TABLE I

GROWTH OF POLYOMA TUMOUR 4198† IN (C57BL × C3H)F_1 MICE: THYMECTOMIZED AND INTACT LITTER MATES

Group	*Treatment*	*Challenging dose* 4198 *cells*	*Relationship of number of mice positive to number of mice challenged*		*Average Polyoma HI titre*
			Ratio	*Per cent positive*	
1	Non-thymectomy + virus	2×10^5	15/69	22	1350
2	Thymectomy + virus	2×10^5	42/49	86	1150
3	Non-thymectomy + tris buffer	2×10^5	25/26	96	< 100
	Thymectomy + tris buffer	2×10^5			

† Tumour 4198 arose in tissue cultured cells from the subcutaneous tissues of a C3Hf/HeN mouse. Polyoma virus was added to the culture and transformation occurred after 16 weeks. These neoplastic cells have been maintained *in vitro* and *in vivo* since 2/2/61 and new antigen has persisted. The *in vitro* cultured line was used for these inoculations. Thymectomies were done at 3 days of age. Numerous and exhaustive tests for detection of polyoma virus have been made and all were negative. (See Ting and Law, 1965.)

mice transforms normal cells to clones of neoplastic cells. The immunologically competent adult recognizes the new cellular antigen as foreign and is capable of rejecting these neoplastic cells in a homograft type of reaction. The animal becomes sensitized to new antigen and, when later challenged with tumour cells containing the same antigen, it quickly and effectively rejects the cells. This capacity of immunized adult mice to reject neoplasms which ordinarily grow progressively and kill the host is known not to be related to circulating antiviral antibodies. Recent observations in this laboratory (Ting, 1966) show that immunization of adult C57BL mice with a virus-free polyoma tumour containing new cellular antigen is as effective as immunization with the virus in achieving refractoriness to a subsequently grafted syngeneic (C57BL) neoplasm of the polyoma type.

That this specific transplantation resistance is due to an immunological reaction which is mediated mainly by lymphoid cells is shown in experiments using thymectomized mice which have received an immunizing dose of polyoma virus (Table I, group 2). These adult mice are incapable of resisting syngeneic tumour cells despite the presence of high levels of antiviral antibody. Treatment of thymectomized mice by subcutaneous thymic grafts is known to restore the capacity to resist tumour challenge (Ting and Law, 1965).

THE THYMUS AND CARCINOGENESIS

It is now apparent that many neoplastic growths contain "tumour" antigens† not present in the host tissues. This applies

† These antigens are those capable of evoking a homograft type of reaction. They are demonstrable only by the presence of resistance to syngeneic tumour challenge in animals immunized with tumour cells containing the antigen, or by the specific virus. Such "tumour" antigens have also been demonstrated in other DNA tumour-inducing viruses such as SV-40 and adenovirus-12 (Habel and Eddy, 1963; Koch and Sabin, 1963; Defendi, 1963; Trentin and Bryan, 1964). It has been shown that one or more new specific antigens ("neo" antigens), present in certain virus-free tumours as well as in non-neoplastic cells infected *in vitro*, react with sera from animals carrying transplanted tumours. This response

particularly to those tumours induced by certain tumour viruses (*vide supra*) and by chemical carcinogens (Prehn and Main, 1957; Old *et al.*, 1962; Klein *et al.*, 1960). In time we hope that this will be found to be true for other neoplasms. It is now also apparent that immunological inadequacy facilitates carcinogenesis in certain experimental situations. These findings imply that antigenic neoplastic cells may periodically arise in the experimental animal and in man but are promptly eliminated or suppressed by the host's adequate immunological mechanisms (see Burnet, 1964).

We will now attempt to evaluate our present knowledge of the relationship of immunological competence and carcinogenesis initiated by several agents. The role of neonatal thymectomy, which leads to an assortment of immunological deficiencies, has been studied in relation to neoplasms induced by polyoma virus and the mammary tumour virus, and in relation to methylcholanthrene (MCA)-induced neoplasms.

Table II shows the results of tumour induction by polyoma virus in several strains of mice normally susceptible or resistant to the oncogenic effects of the virus. A high frequency of neoplasms was observed in all strains thymectomized at three days of age, particularly if virus was given within the first week of life. The common type of neoplasm observed in the refractory C57BL, C3Hf/Lw and F_1 mice was of the parotid gland and was usually bilateral; other salivary glands were neoplastic to varying extents. Strains of mice which were highly refractory developed tumours in their mammary glands and hair follicles as well as in the parotid glands. The highest frequency and multiplicity of

has been determined by complement fixation (CF) reactions (Huebner *et al.*, 1963; Black *et al.*, 1963). The precise relationship of the "neo" antigens to "tumour" antigens is not known. Recently Habel (1965) has shown that tumour antigen of polyoma virus and CF antigens in polyoma-type tumours are not the same; the presence of demonstrable anti-tumour CF antibodies is not necessary for the immunized animal to resist the challenge of a syngeneic polyoma tumour. Neither of these antigens represents or is related to virus antigen.

TABLE II

POLYOMA-INDUCED NEOPLASMS IN SEVERAL STRAINS OF MICE FOLLOWING EARLY THYMECTOMY

Strain	*Group*	*Number of Litters*	*Virus strain*	*Virus concentration*	*Age at virus infection (days)*	*Relationship of number of mice with tumours to number of mice infected*		*Latent period (months)*	
						Ratio	*Per cent infected*	*Mean*	*Range*
C57BL	Thymectomy (3 day)	8	LID-1	2×10^6 PFU	4–7	18/26	73	$2\frac{1}{2}$	$2–4\frac{1}{2}$
	Control					0/15		—	
	Thymectomy (3 day)	15	LID-1	2×10^6 PFU	14–18	21/42	50	3	$2\frac{1}{2}–4$
	Control					0/39		—	
	Thymectomy (1 day)	10	LID-1	2×10^6 PFU	4–7	15/19	78	2	$1\frac{1}{2}–3$
	Control					1/37		$4\frac{1}{2}$	
(C57BL × C3H)F_1	Thymectomy (3 day)	4	LID-1	2×10^6 PFU	10–15	3/11	28	$3\frac{1}{2}$	4–5
	Control					0/8		—	
	Thymectomy (3 day)	7	LID-1	4×10^6 PFU	16–30	4/25	16	$5\frac{1}{2}$	$2\frac{1}{2}–11\frac{1}{2}$
	Control					0/15			
	Thymectomy (1 day)	6	LID-1	2×10^6 PFU	5–6	8/10	80	$3\frac{1}{2}$	$2–5\frac{1}{2}$
	Control					0/16		—	
C3Hf/Lw	Thymectomy (3 day)	5	LID-1	2×10^6 PFU	7–16	9/15	60	$5\frac{1}{2}$	$4–7\frac{1}{2}$
	Control					0/10			
	Thymectomy (3 day)	7	S/M	1×10^7 PFU	4–13	6/21	29	$9\frac{1}{2}$	$8\frac{1}{2}–11\frac{1}{2}$
	Control					0/15		—	
C3Hf/Bi	Thymectomy (3 day)	5	LID-1	2×10^6 PFU	8–14	16/19 †	84	$3\frac{1}{2}$	2–12
	Control					2/10		$5\frac{1}{2}$	4–7
A	Thymectomy (1–5 days)	12	LID-1	2×10^6 PFU	4–6	6/33 ‡	18	$3\frac{1}{2}$	$2\frac{1}{2}–4$
	Control					0/26		—	

† Multiple neoplasms. See text.
‡ Unusual polyoma-type neoplasms within 4-month period. See text.
For description of LID-1 virus, see Ting and Law, 1965.
PFU = plaque-forming units.

polyoma-type tumours were observed in the normally susceptible C3Hf/Bi strain. In these mice although LID-I virus was given late—8 to 14 days of age—multiple neoplasms were observed in most mice thymectomized at three days of age. In addition to the

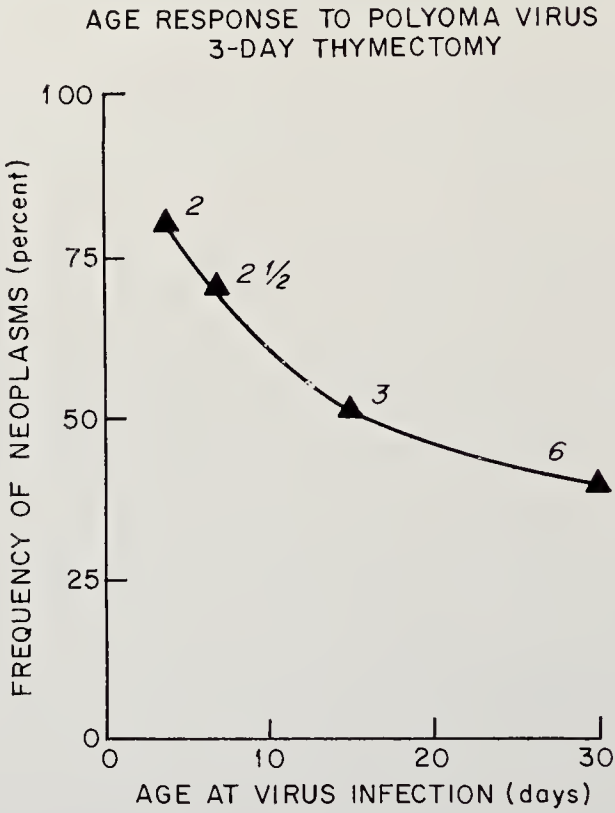


FIG. 2. Frequency of parotid gland tumours in C57BL strain mice thymectomized at 3 days of age. ▲= polyoma virus, (2–4) × 10^6 plaque-forming units, injected at times (days) after birth. Average latent periods (months) are shown in figures above each triangle.

salivary glands, the sites involved were mammary glands, hair follicles, subcutaneous connective tissues, kidney and bone.

The response to polyoma virus of C57BL and F_1 mice thymectomized within 24 hours of birth is shown for comparison in Table II.

TABLE III

EFFECT OF NEONATAL THYMECTOMY AND LYMPHOID CELL RESTORATION ON THE INDUCTION OF NEOPLASMS IN C57BL MICE

Group	*Number of mice*	*Number of mice with parotid tumour*	*Percentage of mice with parotid tumour*	*Latent period (months)*
Thymectomy	43	29	68	3
Thymectomy + spleen cells	45	2	4	4
Thymectomy + lymph node cells	21	3	14	3
Thymectomy + spleen cells from thymectomized donors	28	8	29	$3\frac{1}{2}$
Intact	26	0	—	—

20×10^6 adult cells of each type given intravenously 24 hours after thymectomy.

Polyoma virus, 2×10^6 plaque-forming units given 8–11 days after introduction of adult lymphoid cells.

The usual polyoma-type tumour in C57BL mice involves a single or both parotid salivary glands; other salivary glands were neoplastic to varying extents.

The period of sensitivity of salivary gland tissue to the oncogenic effects of LID-I polyoma virus in C57BL thymectomized mice extended to at least 30 days—the latest age at which virus was introduced in the present experiment (See Table III, and Fig. 2).

Several variant "strains" of polyoma virus, in addition to LID-I (Law and Ting, 1965), have been effective in inducing polyoma-type neoplasms in mice thymectomized at three days of age. S/M, a relatively non-oncogenic small-plaque variant, induced parotid gland tumours in C3Hf/Lw mice although the latent period was longer than with the LID-I strain (see Table II). SE 3049 and KP polyoma virus strains induced salivary gland neoplasms and ameloblastomas of the gingival epithelium respectively in thymectomized C57BL mice. No tumours have been observed in C57BL intact mice inoculated at birth with either of these two variant strains. It is interesting that Malmgren, Rabson and Carney (1964) observed an increased frequency of polyoma-induced neoplasms in thymectomized mice infected early in life with a known "attenuated" strain of polyoma (M-polyoma).

ONCOGENESIS IN THYMECTOMIZED MICE RESTORED BY SEVERAL METHODS

Evidence is now presented to show that interference with the immune mechanism(s) is a necessary prerequisite for successful tumour induction in normally refractory neonatal mice and in refractory adults.

The immunological defects resulting from thymectomy within 24 hours of birth, the lymphoid cell depletion and the disturbed growth (resulting in wasting in some but not all inbred strains of mice) can be prevented by several methods: (a) grafting of newborn thymic tissue; (b) intravenous introduction of adult lymphoid cells from syngeneic donors; or (c) intraperitoneal installation of cell-tight Millipore diffusion chambers containing

neonatal thymic tissue (Miller, 1962; Dalmasso *et al.*, 1963; Parrott and East, 1964; Law *et al.*, 1964).

It has been shown (Law, 1965*a*) that C57BL mice, thymectomized at birth, were restored if given (6 to 12) $\times 10^6$ dissociated syngeneic spleen cells from an adult mouse 48 hours after birth. The restored mice had a normal growth pattern, there was no depletion in lymphoid organs and they had a normal capacity to form haemolysins to sheep erythrocytes. Only 36·6 per cent of the restored C57BL mice had parotid gland tumours compared with 80 per cent of the neonatally thymectomized mice. In this group polyoma virus was administered early—at four to six days of age, that is two to four days after the inoculation of adult spleen cells. If virus is introduced later—eight to eleven days after the introduction of 20×10^6 syngeneic spleen cells—a return to nearly complete refractoriness is observed (Table III). Adult syngeneic cells from lymph nodes are also shown to be nearly as effective as adult spleen cells, but spleen cells from neonatally thymectomized mice are less capable of restoring the capacity to resist polyoma virus oncogenesis.

Table IV shows that other methods of restoration of neonatally thymectomized mice also effectively prevented the induction of the usual polyoma type of neoplasms encountered in thymectomized mice.

In group 1, splenic cells from adult syngeneic donors were effective whether given intraveneously, (6 to 12) $\times 10^6$ cells at four days, or intraperitoneally, 3×10^7 cells at seven and again at nine days. The results of both groups were similar and are combined. In the group in which the mice were thymectomized at three days, the usual stigmata associated with thymectomy performed within 24 hours of birth were not observed. There was little or no evidence of wasting, there was no general lymphoid depletion in organs or peripheral blood and haemolysin titres to sheep erythrocytes were similar to those of intact litter mates; C57BL mice thymectomized at three days were also capable of rejecting

TABLE IV

RESPONSE OF THYMECTOMIZED AND RESTORED C57BL MICE TO LID-I STRAIN OF POLYOMA VIRUS

	Group	Number of litters	Relationship of number of mice with tumours to number of mice		Latent period (months)		Remarks
			Ratio	Per cent	Mean	Range	
1	Thymectomy at 3 days	21	20/31	65	$2\frac{1}{2}$	2–4	LID-I polyoma virus injected subcutaneously at 13–18 days 4×10^6 PFU/mouse
	Thymectomy + dissociated spleen cells		2/29	7		5,7	
	Sham and intact*		0/42		—		
2	Thymectomy at 1–3 days	12	9/24	38	6	3–12	Polyoma virus injected subcutaneously at 20–30 days 4×10^6 PFU/mouse
	Thymectomy + Millipore chambers containing neonatal thymus at 14 days		1/8	12·5	$1\frac{1}{2}$	—	
	Intact		0/20		—		
3	Thymectomy at 1 day	7	9/12†	75	$2\frac{1}{2}$	2–4	Polyoma virus injected at 14 days 2×10^6 PFU/mouse
	Thymectomy + syngeneic thymic graft at 1 day		2/12‡	17	—	$3\frac{1}{2}$,9	
	Intact		0/30		—		

* No differences in response between sham-operated and intact mice; other controls in groups 1 and 2 were therefore not given a sham operation.

† Two mice died of wasting before tumour induction.

‡ One mouse died wasted but with a parotid gland neoplasm at $3\frac{1}{2}$ months of age.

PFU = plaque-forming units.

allogeneic skin grafts. Thymectomy at this time still has an effect on the development of immunological reactivity to certain antigenic stimuli (Miller, 1963).

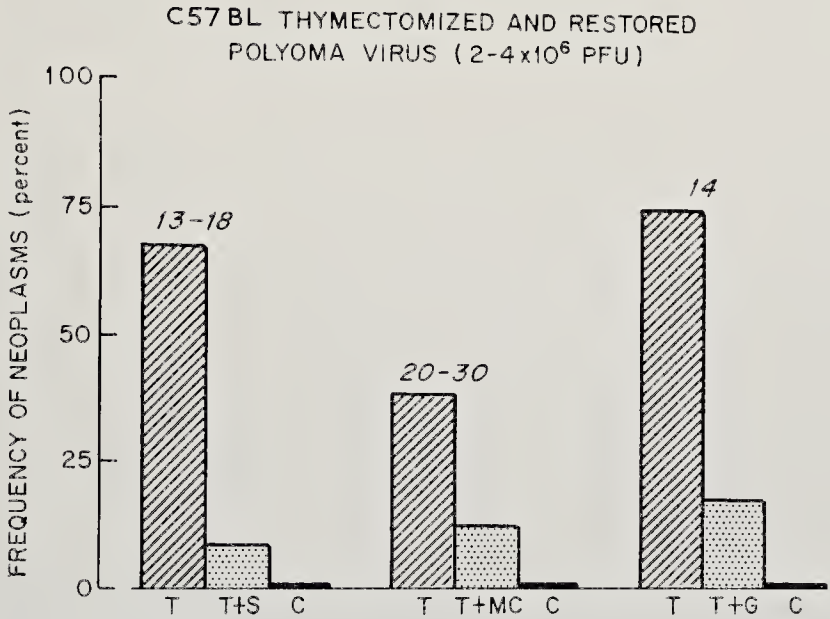


FIG. 3. Frequency of parotid gland neoplasms in the 3 groups of C57BL mice, thymectomized at 3 days and restored by several methods. T= thymectomy. C=intact. T+S=thymectomized mice restored with adult syngeneic spleen cells. T+MC=thymectomized mice bearing Millipore diffusion chambers with thymic tissue. T+G=thymectomized mice bearing syngeneic thymic grafts. Age (days) at which LID-I polyoma virus injected is given above each group. PFU=plaque-forming units.

The Millipore chambers were recovered at twelve months from the restored tumour-negative mice in group 2. In the chambers, the mass of persisting tissue was principally necrotic but, in many, viable tissue was observed in the form of fibroblasts and nests of reticular-epithelial tissue.

Fig. 3 shows the relative effects of restoration by the three methods employed.

VIRUS GROWTH AND ANTIBODY FORMATION IN THYMECTOMIZED MICE

It is unlikely that the striking increase in susceptibility to oncogenesis by the polyoma virus after thymectomy is the result of non-specific factors affecting the general health of the

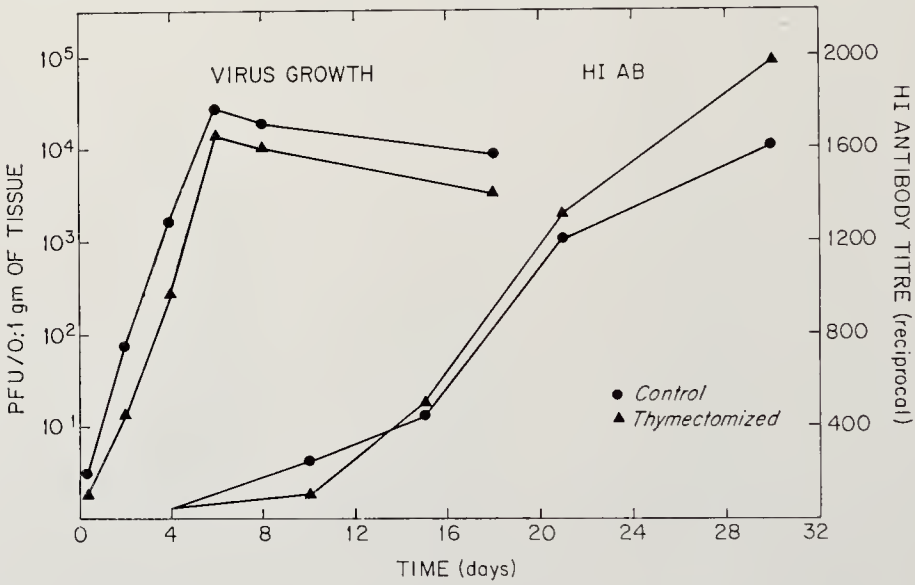


FIG. 4. Growth curves for LID–I polyoma virus in intact C57BL mice and in animals thymectomized at 3 days. Virus injected (1×10^5 PFU) at 7 days. Three mice in each group were killed at various times from 4 hours to 18 days after injection for determination of virus concentration. Points represent average concentrations of virus in kidney, liver and salivary glands (9 mice for each point). Virus assays done according to the method of Dulbecco and Freeman (1959). HI AB = haemagglutination-inhibition antibody. Six mice in each group received the same input of virus and titres were determined in the same mice from 4 to 3 days later. PFU = plaque-forming units.

mice. The mice thymectomized at three days were in excellent condition and showed no evidence of the trophic disturbances characteristically seen when such mice are thymectomized on the day of birth.

The absence of thymic tissue might enhance virus growth and lead to an earlier and greater concentration of virus in the target

organs. The highly oncogenic S-polyoma virus, for example, showed a titre that was a thousand times greater at five days after early infection than did the M-polyoma, low-oncogenic variant (Friedman and Rabson, 1964). It is clear from Fig. 4, which shows the growth curves of the virus in thymectomized and intact C57BL mice, that growth of the virus is not a significant factor in the increased susceptibility of neonatally thymectomized mice; no significant differences were found in HI†-antibody titres determined periodically from day 4 to day 30. These results, which relate to virus growth and antibody formation, are similar to the results observed in thymectomized and intact Swiss-Webster mice in their response to lymphocytic choriomeningitis (LCM) infection (Levey *et al.*, 1963), except that in the experimental situation described by Levey thymectomized mice were more refractory to the lethal effects of LCM than their intact litter mates. Virus growth patterns and antibody formation however were similar in thymectomized and intact mice.

SPONTANEOUS INFECTION IN THYMECTOMIZED MICE LEADING TO TUMOUR INDUCTION

The studies recorded here were initiated to examine the effect of early thymectomy on the development of spontaneous mammary cancer in the females of a high mammary tumour strain of mice and at the same time to investigate the effect of thymic removal on methylcholanthrene (MCA)-induced fibrosarcomas in their male litter mates (Law, 1965*b*).

C3H/HeN strain mice from the National Institutes of Health's colony were used. These mice carry the mammary tumour milk agent. They were thymectomized at three days of age. The completeness of thymectomy was checked by serial sections of tissue obtained at necropsy from the upper mediastinal area. At least two members of each litter were left as intact controls.

† HI equals haemagglutination-inhibition.

Litters were raised by their own mothers and at four to five weeks were separated according to sex, but with thymectomized and intact mice remaining together, six to eight mice per cage. The mice were housed in an animal room containing other experimental mice some of which had received polyoma virus parenterally. Females received no further treatment; males received 0·1 mg. of 3-MCA dissolved in 0·2 ml. tricapyrlin, subcutaneously at eight weeks. The mice were examined daily for neoplastic growths. Unexpectedly, an untreated female developed bilateral parotid gland tumours at the age of four and a half months. At this time polyoma HI antibodies (see Eddy *et al.*, 1958) were determined in the sera of both males and females. The members of six out of fifteen litters of the untreated females showed HI antibodies ranging from 1:400–1:12,800; an HI-antibody survey taken when the mice were two months old showed that they all had titres of < 1:100 (HI-negative). Later, 14 additional females presented with a polyoma type of neoplasm but these all came from among the 23 thymectomized mice in the six HI-positive litters. All intact mice that showed infection with the polyoma virus remained, nevertheless, tumour free as did all members of the nine HI-negative litters (see Table V).

The neoplasms, grossly, were confined to one or both parotid glands. Histological examination also revealed numerous neoplastic areas in other salivary glands. The histological appearance of these neoplasms was similar to that described by Law, Dunn and Boyle in 1955. There were poorly-defined tubular structures with a tendency to loss of epithelial character and gain of spindle-cell features.

No differences were observed in the frequency or latent period of MCA-induced fibrosarcomas between the male mice thymectomized at three days and their intact litter mates. Eighty-one per cent (47 out of 58) of the thymectomized mice and 79 per cent of the controls (23 out of 29) had neoplasms; the average latent periods (after introduction of carcinogen) were 76 and 74

TABLE V

POLYOMA-TYPE NEOPLASMS AND MAMMARY ADENOCARCINOMA IN VIRGIN C3H/HeN FEMALE MICE: THYMECTOMY AT THREE DAYS AND ROOM INFECTION

	Polyoma HI antibodies (according to litter)			*Relationship of number of mice with neoplasms to total number of mice*		*Latent period (months)*	
Group	*Positive*	*Negative*	*Titre*	*Ratio*	*Per cent*	*Mean*	*Range*
Thymectomy	6		1:4500†	15/23	65·2	6·8	4–11
		9	<1:100	0/34			
				15/57	26·3		
Intact	6		1:600	0/15	0	—	
		9	<1:100	0/16	0	—	
				0/31	0		
Thymectomy	Mammary adenocarcinomas show no relationship to polyoma HI antibodies			19/42	45·2	12·7	10–16
Intact				21/31	67·7	10·6	8–16

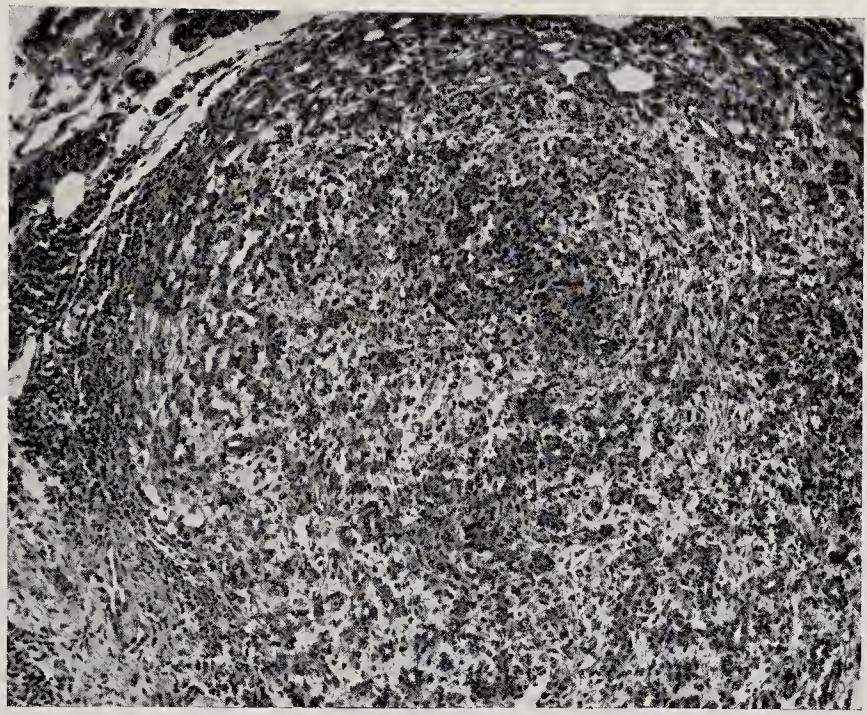
† The higher average titre among thymectomized mice resulted from excessively high titres in several mice with tumours. If these are excluded, average titres are similar in thymectomized and intact mice.

days respectively. Among the C3H/HeN males which had been thymectomized at three days, only 23 were at risk to polyoma virus infection. The risk occupied from four and a half to six and a half months during part of the period when parotid gland tumours appeared in the female litter mates. Only one male mouse developed parotid gland neoplasms, and this was at six months of age, although more than half the male mice had evidence of polyoma infection.

Mammary tumours are now appearing in the remaining C3H females older than seven and a half months. These tumours have the typical morphology of such neoplasms when they arise in mice with the Bittner milk agent, reproducing mammary acini in many areas. The tumours are mostly classified as adenocarcinomas. Histological comparison of these adenocarcinomas with a typical parotid gland neoplasm is shown in Fig. 5. The mammary tumours occurred both in polyoma HI-positive and HI-negative females. After sixteen months' observation the frequency is lower and the latent period longer among the thymectomized mice (Table V). At one year there was a pronounced difference in frequencies: 7 out of 42 (17 per cent) in thymectomized females and 21 out of 31 (52 per cent) in intact females. These findings are similar to those reported among breeding females of another C3H subline (Martinez, 1964).

The pattern of spontaneous polyoma virus infection in our inbred mouse colonies in which experimentally inoculated mice were housed, has been reported elsewhere (Rowe *et al.*, 1959). A unique feature of the spontaneous infection pattern was the nearly complete absence of virus, as determined by the presence of HI antibody, up to two to three months of age, but the increasing frequency of this antibody with age beyond three months. Although a high frequency of polyoma virus infection was observed in some strains of mice, the occurrence of polyoma-type tumours was rare. Table VI compares the infection rate in C3H mice, for 1959 and in mice used in the present study.

(*a*)



(*b*)

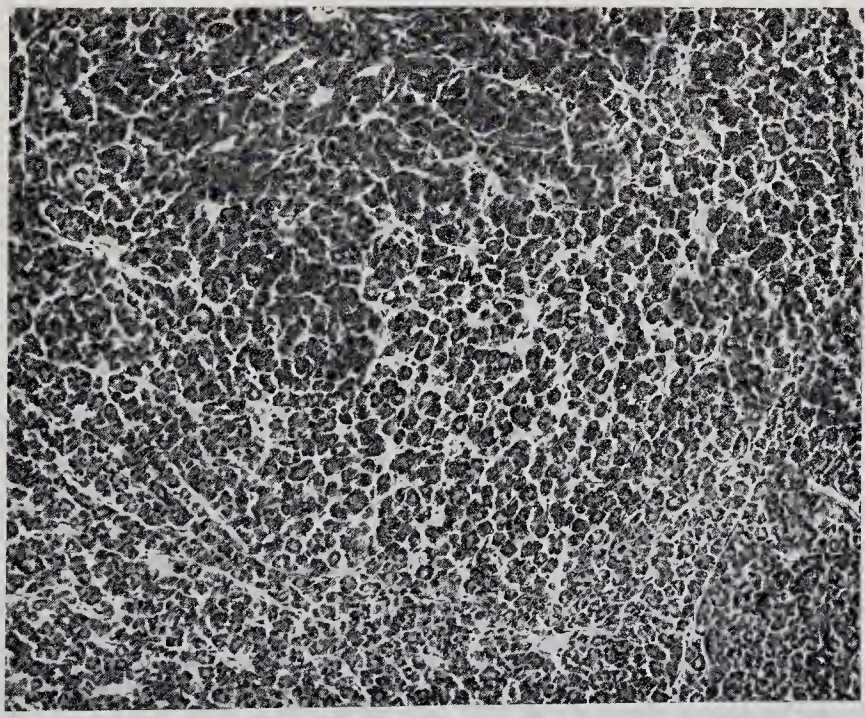


FIG. 5. (*a*) Parotid gland neoplasm in a C3H female mouse, 7 months of age. Mouse thymectomized at 3 days of age. (× 140). (*b*) Mammary adenocarcinoma at L-4 in a 10-month-old intact C3H female mouse. (× 140).

TABLE VI

INCIDENCE OF HI ANTIBODY TO POLYOMA VIRUS (ROOM INFECTION)

	Antibody incidence by age group					
Age in months	1–3		4–9		> 10	
Group	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*
1959—Colony	4/95	4	61/230	27	88/192	46
1964—C3H†	0/40	0	23/53	44	28/53	53

† Includes a sample of both males and females used in the present study. At 12 months the number 1 frequency of the remaining mice had reached nearly 70 per cent. All except one parotid tumour, however, appeared before 9 months.

Probably the only way in which the mouse, infected through natural means as an adult, can develop a polyoma-induced neoplasm is by the chance occurrence of an event which reduces its immunological competence. Thymectomy early in life is an effective method of accomplishing this and is apparently more effective than total-body irradiation. C3H mice in this same colony which received X-irradiation—either a single dose of 400 r. or 150 r. on four occasions—and 80 per cent of which had high levels of HI antibodies, have failed to develop polyoma-type neoplasms. C3H mice, thymectomized within 24 hours of birth but restored with adult syngeneic lymphoid cells (see Trainin, Law and Levey, 1965) and housed in the same laboratories, have remained free from tumours; 31 C3H mice, reconstituted with spleen cells, lymph node cells or bone marrow, were in this group and all had high levels of polyoma HI antibody when tested at nine to twelve months of age.

These experimental results are also in accord with the concept that the basis of resistance to polyoma virus oncogenesis is a cellular immunity of the homograft type directed against virus-specific new "tumour" antigen contained in the neoplastic cells. In the immunologically disturbed animal these antigens are not capable of provoking an effective immunological attack on the cells transformed to neoplasia by virus. These clones of cells therefore grow progressively into frank neoplasms.

It remains to be determined how important a part this defence mechanism—homograft rejection—plays in the elimination of malignant cells that may arise from time to time throughout the life of higher organisms. Our results with virus-induced mammary cancer and with MCA-induced fibrosarcomas were un-

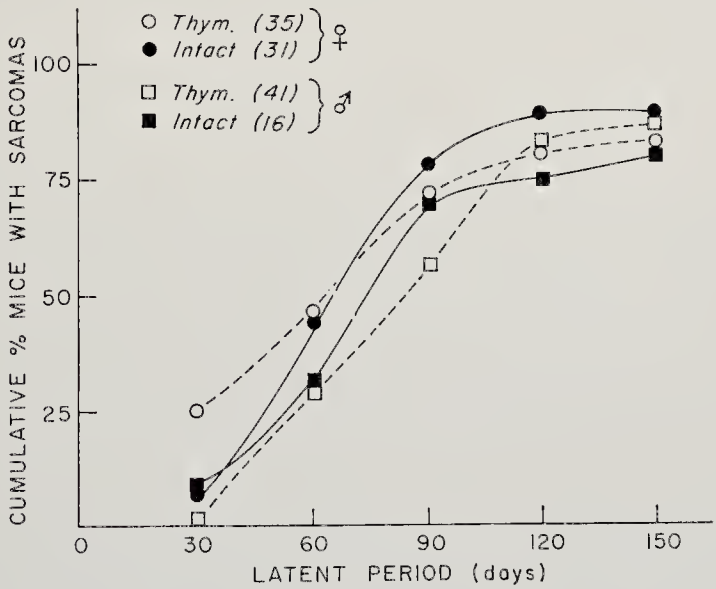


FIG. 6. Cumulative frequencies of fibrosarcomas in C3H/HeN male and female mice following injection of 50 μg. MCA at 6–8 weeks. Latent period = time following introduction of carcinogen. See text for frequencies and latent periods of C3H males in a separate group. Numbers in parentheses = total numbers in each group, intact and thymectomized.

expected in view of the evidence that both types of neoplasms contain "tumour-specific" antigens (Weiss, Franklin and DeOme, 1964; Prehn and Main, 1957; Klein *et al.*, 1960). These antigens should be beneficial to the host in evoking homograft reactions.

Since the results of Defendi and Roosa (1964) and of Grant and Miller (1965) gave suggestive evidence that neonatal thymectomy shortens the latent period and influences the frequency of MCA-induced fibrosarcomas, our earlier experiments were repeated in

both C3H and C57BL strains of mice thymectomized at three days. The dose of MCA was reduced to 50 μg. per mouse. We found no differences in the latent periods, frequency of induced neoplasms or growth potential of the induced neoplasms in thymectomized mice and their sham-thymectomized litter mates. The results in C3H males and females are shown in Fig. 6.

DISCUSSION

The genetically controlled variation to the induction of polyoma virus tumour in mice appears to be determined by one or a few genes and is not a property of individual target cells or of individual cells which influence viral growth patterns. It thus differs from the susceptibility and resistance to virus infection studied by Bang and Warwick (1960), Goodman and Koprowski (1962) and Rubin (1965). Isolated organ cultures of C57BL mice transform readily to neoplasia in the presence of polyoma virus; resistance is therefore a characteristic of the intact animal.

The most plausible explanation for resistance to polyoma virus oncogenesis is related directly to the immunological capacity of the host animal; cellular immunity of the homograft type invoked against virus-specific "tumour" antigen, contained in neoplastic cells, is involved. In the immunologically disturbed animal (following neonatal thymectomy) these "tumour" antigens are not capable of invoking an effective immunological attack on clones of cells transformed to neoplasia by polyoma virus. Neoplastic clones, therefore, grow progressively into frank neoplasms. In the immunologically competent animal, either intact or restored, neoplastic conversion occurs but the transformed cells are aborted by a successful homograft type of reaction. Immunological restoration was accomplished in the thymectomized C57BL mouse by all three of the methods used to the extent of permitting adequate homograft rejection and

thus repressing the origin of the neoplastic cells. C57BL mice are known to become immunologically competent soon after birth (Möller, 1961).

The immunological deficit resulting from thymectomy at three days of age which allows tumour induction by polyoma virus is quite subtle and not easily recognized on gross examination. Little or no lymphoid cell depletion was noted and these mice (C3H and C57BL) were quite capable of rejecting allogeneic skin grafts, and of forming haemolysins and agglutinins to sheep erythrocytes and HI antibodies to polyoma virus normally. C57BL female mice thymectomized at three days, however, were less capable of rejecting male skin than their intact litter mates. Twelve thymectomized females rejected male skin at an average time of 68 days whereas all fifteen intact control litter mates had rejected skin grafts by 30 days.

These results suggest that the polyoma virus-specific "tumour" antigen is functionally similar to a minor histocompatibility antigen.

It should be re-emphasized that resistance and susceptibility to tumour induction by polyoma virus is unrelated to viral growth patterns and to humoral antibody production to virus. The patterns were nearly identical in intact and thymectomized C57BL mice. These findings also suggest that a difference in interferon production is not a major factor in increased susceptibility to polyoma virus.

Thymic tissue *per se* is a necessary antecedent of resistance to the induction of polyoma virus tumours to the extent that it provides for the maturation of immunological faculties and consequently prevents the progression of neoplastic clones of cells induced by the virus. If many tumours contain "tumour" antigens foreign to the host, it is likely that the host can respond immunologically against these antigens and destroy or inhibit emerging clones of neoplastic cells. Neonatal thymectomy should therefore induce an increased susceptibility to the develop-

ment of such tumours. The generality of the influence of thymectomy is not yet determined. Early thymectomy in the rat and hamster is associated with an increased sensitivity to polyoma virus (Vandeputte *et al.*, 1963; Defendi and Roosa, 1964). Neonatal thymectomy in the Syrian hamster (*Mesocricetus auratus*) did not effect the induction of gliomas or fibrosarcomas by RSV (Rabotti and Law, 1965), nor was there an increased yield or lowered latent period in mammary carcinoma induced by the Bittner virus or in MCA-induced fibrosarcomas, as has been revealed in this study.

It has been pointed out that the striking sensitivity of newborn animals to tumours induced by the polyoma virus may be related to the fact that the most responsive tissues are still in an embryonal and undifferentiated state (Ham *et al.*, 1962; Dawe, Law and Dunn, 1959). Neoplastic transformation does not usually occur in mice and hamsters older than 20 days since differentiation of the susceptible tissues has already occurred at this time. The results discussed here, showing that C3H mice infected "spontaneously" as adults can readily develop tumours providing an immunological deficit is created through neonatal thymectomy, appear to rule out such a concept.

SUMMARY

Thymectomy at three days of age in several inbred strains of mice and in an F_1 hybrid resulted in strikingly increased frequencies of neoplasms following parenteral injection of various "strains" of polyoma virus.

Age susceptibility to the oncogenic effects of the virus was extended to the adult period.

The usual stigmata associated with thymectomy at birth were not observed in the mice thymectomized at three days. Nevertheless, the methods used to restore immunologically deficient neonates were also effective in restoring the capacity to resist tumour induction by polyoma virus.

Growth curves of polyoma virus in kidney, salivary glands and liver were found to be similar in the thymectomized mice and their intact litter mates. Haemagglutination-inhibiting (HI) antibodies determined periodically up to 30 days were also similar in both groups.

A high frequency of parotid gland tumours was observed in C3H female mice thymectomized at three days. In these mice "spontaneous" infection with polyoma virus occurred between two and four months. Intact litter mates similarly infected have remained free of polyoma-type tumours. In contrast, typical mammary adenocarcinomas appeared later and in lower frequency in thymectomized C3H females and the incidence and latent period of MCA-induced fibrosarcomas was not influenced by thymectomy.

The results are discussed in terms of a concept involving virus-specific "tumour" antigens and the immunological status of the animal.

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THE NATURE AND REGULATION OF LYMPHOPOIESIS IN THE NORMAL AND NEOPLASTIC THYMUS

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THE thymus cortex is composed of dense aggregates of lymphoid cells which exhibit intense mitotic activity. Many investigators have reported that thymic lymphoid cells have higher mitotic indices than other lymphoid cells. These estimates were made either from sections (Kindred, 1938) or cell smears (Andreasen and Christensen, 1949; Metcalf and Nakamura, 1962; Metcalf, 1964*a*) and were based on mitotic counts per tissue area or mitoses per total number of primitive lymphoid cells. All three types of data in the rat, mouse and human indicate that thymic mitotic indices are about ten times higher than subcutaneous lymph node, and five times higher than mesenteric node or Peyer's patch, mitotic indices.

Recently we have studied lymphopoiesis in normal AKR and C57BL mice following a single intravenous injection of 5 μc/g. of tritiated thymidine ([^{3}H]thymidine). The data for the normal AKR thymus have been published in full elsewhere (Metcalf and Wiadrowski, 1966). In summary, AKR thymus cell smears were found to contain approximately 1 per cent large lymphocytes (cell diameter $> 11\,\mu$), 10 per cent medium lymphocytes (cell diameter 7–11 μ) and 89 per cent small lymphocytes (cell diameter $< 7\,\mu$) (Fig. 1). Data from corrected grain-count curves,

and from a combination of percentage labelling data at 1 hour and mitotic labelling curves, gave the cell cycle estimates shown in Table I. Large lymphocytes appeared to divide every 6·8 hours and medium lymphocytes every 8·2 hours. Short as these cell cycle times are, they are no shorter than those reported in recent

FIG. 1. Thymic cell smear from 2-month-old AKR mouse, 1 hour after labelling with [^{3}H]thymidine. Note labelling in large (L) and medium (M) lymphocytes and absence of labelling in small lymphocytes (S). Medium lymphocyte in metaphase (Me). [^{3}H]thymidine dose: 5μc/g., exposure time 18 days. Giemsa (× 800).

[^{3}H]thymidine studies on cells from the germinal centres of lymphoid follicles of the spleen (6–7 hours) in antigenically stimulated rats and mice, by Fliedner and co-workers (1964) and Hanna (1964). Labelling data from our laboratory on C57BL mice also suggested that primitive cells from the thymus and lymph nodes showed a smaller difference in proliferative rates than has been indicated by earlier measurements of mitotic indices.

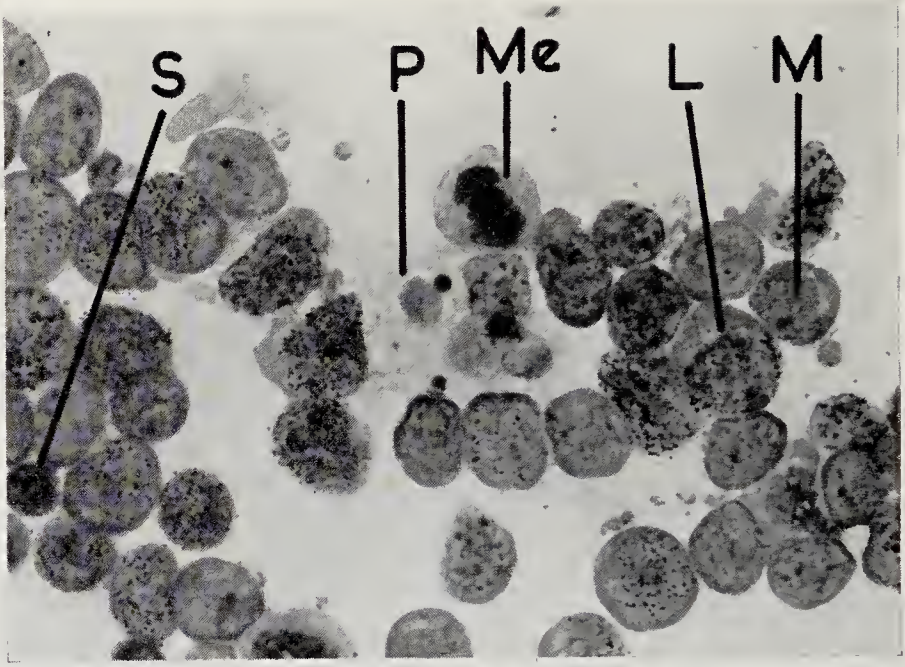

FIG. 2. Thymic lymphoma cell smear from AKR mouse, 6 hours after labelling with [^{3}H]thymidine. Most cells are large (L) and medium (M) lymphoma cells; small lymphoma cells (S) are rare. Note phagocytic reticulum cell (P) containing three pyknotic lymphoma cells and cell debris. Medium lymphoma cell in metaphase (Me). [^{3}H]thymidine dose: 5μc/g., exposure time 91 days. Giemsa (× 800).

TABLE I

CELL CYCLE TIMES FOR LARGE AND MEDIUM LYMPHOID CELLS IN THE NORMAL AKR THYMUS AND IN THYMIC LYMPHOMA TISSUE

Tissue	*Cell type*	*Length of cell phase (hours)*			*Total cell cycle time (hours)*
		G_1	S	G_2+M	
Normal 2-month-old AKR thymus	Large	—	5·5	1·3	6·8
	Medium	1·4	5·5	1·3	8·2
AKR thymic lymphoma	Large	—	6·0	1·6	7·6
	Medium	12·0	6·0	2·2	20·2

G_1 = Presynthetic. S = DNA synthesis period. G_2+M = time for mitosis. Data based on 60 normal and 65 leukaemic mice.

The radioautographic data in AKR and C57BL mice indicated an arithmetical accumulation of new small lymphocytes in the thymus, lymph nodes and blood, suggesting that small lymphocytes arise from the asymmetrical division of primitive lymphoid cells—probably the medium lymphocytes. Small lymphocytes

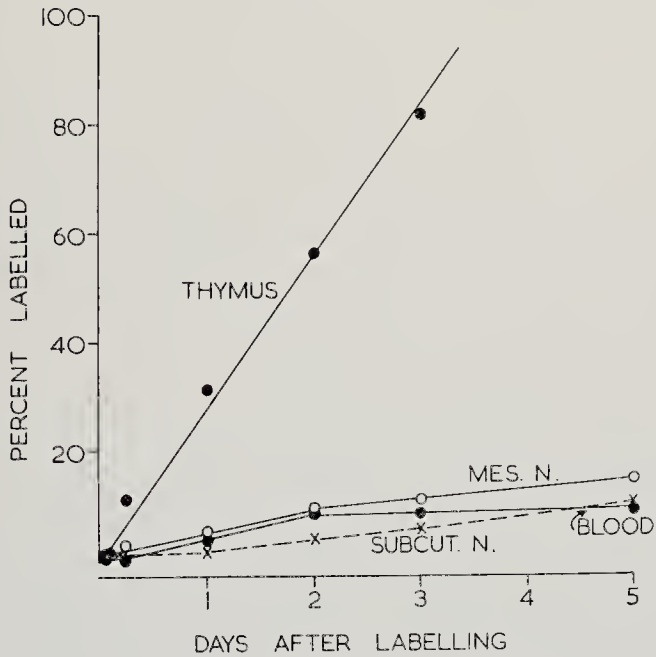

Fig. 3. Accumulation of labelled small lymphocytes in various lymphoid organs in 3-month-old C57BL mice after single intravenous injection of 5μc/g. of [^{3}H]thymidine. Each point represents the mean values for 4 mice. Subcut. N = subcutaneous node. Mes. N. = mesenteric node.

were replaced by new labelled cells at a greater rate in the thymus than in lymph nodes or blood (Fig. 3). Comparison of the present data with that of Osmond and Everett (1964) on the production of small lymphocytes in the bone marrow, indicates that the renewal of small lymphocytes in the thymus is also more rapid than their renewal in the bone marrow. These findings suggest that lymphopoiesis is more rapid in the thymus than elsewhere.

However, in view of the direct radioautographic data on proliferative rates of primitive cells in lymphoid organs, it now seems clear that the slower rate of formation of small lymphocytes in the lymph nodes of C57BL mice compared with the rate in the thymus of these mice results from factors not wholly related to proliferative rates of primitive cells: (a) There is a smaller percentage of primitive cells in the lymph node (0·5 per cent large and 2·6 per cent medium cells in the lymph node compared with 1·4 per cent large and 7·2 per cent medium cells in the thymus). (b) Many of the cells in the lymph node that are typed on cytological grounds as primitive "lymphoid" cells apparently are not concerned with the generation of small lymphocytes but with the generation of other cell types, for example, plasma cells. (c) Many mitotic cells in germinal centres may not give rise to viable offspring (Fliedner *et al.*, 1964).

It should also be appreciated that the mitotic activity of lymphoid cells in the spleen and lymph nodes is almost totally dependent on antigenic stimulation. In the germ-free animal mitotic activity is extremely low in these tissues (Thorbecke *et al.*, 1957; Gordon, 1959) whereas, after recent antigenic stimulation, the mitotic activity of primitive cells (at least in selected germinal centres) equals that present in either the germ-free or the normal thymus. In so-called "normal" animals which are subjected to sporadic low-level antigenic stimulation in the course of their natural exposure to various organisms and antigens, an intermediate situation appears to exist.

An additional comment needs to be made on the significance of the different percentages of primitive lymphoid cells in various lymphoid organs. This might suggest that the pattern of differentiation of lymphocytes differs in different lymphoid organs—specifically, that more lymphoid cells tend to remain primitive in the thymus than in other lymphoid organs. However the true basis for this difference in percentages of primitive lymphoid cells in the various organs is more likely to be simply the differing

life-spans of the progeny of the small lymphocyte. Where small lymphocytes have a long life-span, for example in the lymph node, this inevitably leads to an increased ratio of small lymphocytes to primitive cells, and this situation is reversed in the thymus.

We no longer feel that the thymus exhibits a uniquely high mitotic activity in its primitive lymphoid cells. The unique feature of lymphopoiesis in the thymus is that it is not dependent on antigenic stimulation—the primary stimulus for lymphopoiesis in the spleen and lymph nodes. Associated with this lack of dependence of thymic lymphopoiesis on antigenic stimulation is the well-known absence of lymphoid follicles and germinal centres from the thymus cortex and the rarity of plasma cells (less than 0·1 per cent) in the normal thymus.

MODEL OF LYMPHOPOIESIS IN THE THYMUS

A detailed model has been proposed for lymphopoiesis in the thymus cortex (Leblond and Sainte-Marie, 1960). Our [^{3}H]thymidine data support this model in broad outline, but a more detailed interpretation of the data is precluded because, after initial labelling, primitive cells are able to reutilize label made available by the breakdown of previously labelled cells. It has not, therefore, been possible to determine whether subclasses or generations exist within each of the three classes of thymus cells as proposed by Leblond and Sainte-Marie.

Our current concept of the general details of thymic lymphopoiesis is derived from [^{3}H]thymidine data and repopulation data and is summarized in Fig. 4. Studies using T6 marker chromosomes in parabiotic mice (Harris *et al.*, 1964) and thymus grafts (Metcalf and Wakonig-Vaartaja, 1964) indicate that most primitive cells in the thymus are continuously replaced by new cells entering it from the circulation. This replacement process is slow (possibly of the order of 1 per cent per day) and, unlike the similar replacement processes in the spleen and lymph nodes,

thymic stem-cell replacement appears to be incomplete. There seems to be a fraction (up to 25 per cent) of the primitive thymic lymphoid cells which is either permanently self-replicating or, more likely, replaced by new cells of endogenous origin. These latter cells may originate from epithelial cells, as postulated for lymphopoiesis in embryonic life (Auerbach, 1961), or from dedifferentiated, small, thymic lymphocytes. It is likely that the

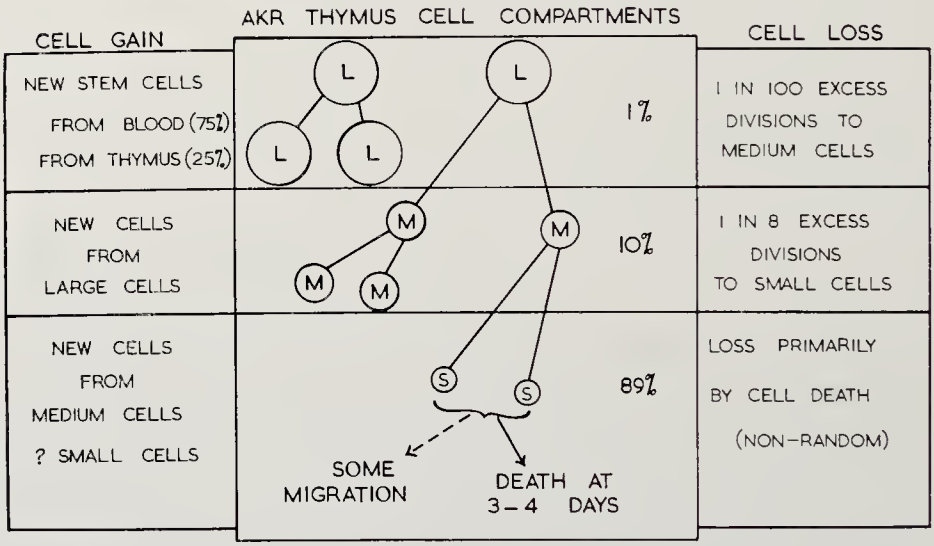

FIG. 4. Schematic diagram of the three lymphoid cell compartments in the normal AKR thymus. Factors involved in cell gain and cell loss for the three compartments are shown.

new primitive cells enter the cell system at the level of the most primitive cell type (the large lymphocyte or, possibly, the reticulum cell). The division of large lymphocytes is asymmetrical—probably alternate cells give rise to two large lymphocytes or to two medium lymphocytes. This asymmetrical division apparently is not exact as it produces a slightly larger number of medium lymphocytes than large lymphocytes. This tends to lead slowly to a deficit of large lymphocytes which is made good by incoming new cells becoming large lymphocytes. Division

of medium lymphocytes also is basically asymmetrical: both medium and small lymphocytes are produced but, again, the medium lymphocytes tend to produce more small lymphocytes (daughter-type) than medium lymphocytes (parental-type). The deficit of medium lymphocytes so created is made good by the new medium lymphocytes produced by large lymphocytes. From the proportions of large to medium lymphocytes and their relative division rates, it can be calculated that roughly one new medium lymphocyte of large-lymphocyte origin is needed per eight divisions of the medium lymphocytes to maintain a constant number of medium lymphocytes.

Small lymphocytes are produced for the most part by asymmetrical division of medium lymphocytes. Some mitotic activity may be possible in small lymphocytes, however, as 2 per cent of these cells directly incorporate [^{3}H]thymidine, but this would constitute only a minor contribution to the production of small lymphocytes. It is even possible that some of the small lymphocytes incorporating [^{3}H]thymidine may be cells in the process of dedifferentiating to large lymphocytes (cf. the behaviour of small lymphocytes *in vitro* after exposure to phytohaemagglutinin) and may represent blood-borne immigrant cells in the process of replacing the population of large lymphocytes.

The rate at which new thymic small lymphocytes are generated in the thymus suggests that they have a mean intrathymic life-span of 3–4 days (Fig. 3). Since the weight of the adult thymus remains constant over relatively long periods, cell death in the organ or migration from the organ must equal new cell production. If it is assumed that most small lymphocytes arise by the eight-hourly division of medium lymphocytes, an experimental curve can be constructed for the increase with time in the population of labelled small lymphocytes following the administration of [^{3}H]thymidine, based either on random loss or age-related loss. The random loss curve differs widely from the observed labelling pattern which, instead, fits almost exactly the situation where

cells remain the full 3–4 days in the thymus before loss. Craddock and co-workers (1964), on the basis of chemical determinations of [^{3}H]thymidine DNA in the rat thymus, have shown that total [^{3}H]thymidine levels remain constant in the thymus for 3–4 days after labelling, in contrast to the progressive loss of label in the lymph nodes which commences immediately after labelling. We have confirmed this observation by making estimates of total thymic grain counts at intervals after a single intravenous injection of [^{3}H]thymidine. Total thymic grain counts were

TABLE II

TOTAL THYMUS GRAIN COUNTS IN TWO-MONTH-OLD AKR MICE AT VARIOUS INTERVALS AFTER LABELLING WITH 5 μc/g. TRITIATED THYMIDINE

Time after labelling (hours)	*Number of mice*	*Total grain count† in different cell populations*			*Total thymus grain count*
		Large	*Medium*	*Small*	
1	6	222	680	163	1065
2	4	238	895	183	1316
6	4	117	510	535	1162
24	4	32	165	930	1127
48	4	17	83	1370	1470
72	4	24	104	1160	1288
96	4	19	90	650	759

† Total grain count = per cent of cell type × per cent labelling × corrected mean grain count of labelled cells. Thymus weight and percentage cell distribution constant between 1 and 96 hours. Grain counts corrected to 10μc/g., 20 days exposure time.

determined by adding the total grain counts of large, medium and small lymphocytes (that is, percentage large lymphocytes × percentage labelling × corrected mean grain count); the thymus weight and percentage of primitive cells being constant between days 0–5 after labelling. Table II shows that the total thymic grain count remained remarkably constant up to three days and then started to fall. If there had been any significant random loss of small lymphocytes from the thymus during the first three days this should have shown as a substantial fall in the total grain count of the small lymphocytes.

These observations make it likely that random loss of small lymphocytes does not occur from the thymus, but rather that cells tend either to die or to migrate from the thymus at the end of their intrathymic life-span of 3–4 days.

DO THYMIC LYMPHOCYTES LEAVE THE THYMUS?

Since the thymic population of small lymphocytes is renewed at such a rapid rate while the size of the organ remains constant, it has been tacitly assumed by many that these cells must be exported in large numbers, presumably to other lymphoid organs. Many investigators have demonstrated that suspensions of thymic lymphoid cells, when injected intravenously, will seed in the spleen and lymph nodes (Fichtelius, 1960), but do such experiments prove that lymphocytes normally leave the thymus? Presumably, if one carefully injected liver-cell suspensions intravenously, liver cells could be demonstrated to lodge in various organs and some might even proliferate there, but this does not prove that liver cells normally migrate from the liver.

There have been two attempts recorded to label thymic lymphocytes *in situ* with [^{3}H]thymidine by direct injection of the isotope into the thymus in such a way as to cause minimal labelling of lymphocytes elsewhere in the body (Nossal, 1964; Murray and Woods, 1964). Both investigations revealed that heavily labelled lymphocytes (presumably thymic in origin) appeared subsequently in peripheral lymphoid organs. However, the number of such "migrant" cells was surprisingly small in relation to the production rate of thymic cells, suggesting that perhaps the majority of thymic lymphocytes do not leave the thymus.

We have approached this question by studying lymphocyte labelling patterns following [^{3}H]thymidine administration to C57BL mice, each grafted one month previously with 12–48 one-day-old C57BL thymus glands. It was found that labelling patterns in the thymus grafts were identical with those in the

normal thymus and that by the third day after labelling up to 90 per cent of the host thymus and thymus-graft lymphoid cells were labelled. At this time only up to 10 per cent of the lymphocytes in the subcutaneous and mesenteric lymph nodes were labelled. Subsequent study of these thymus-grafted animals up to 14 days after labelling failed to reveal any increase over control values for normal C57BL mice in the percentage of labelled lymphocytes in the blood or the subcutaneous or mesenteric lymph nodes (Matsuyama, Wiadrowski and Metcalf, 1966). In these thymus-grafted mice the total of thymus graft and host thymus weights varied from 300 to 1250 mg. compared with a total lymphoid organ weight of the host thymus of 250 mg. If, as indicated by the labelling patterns, thymic populations of small lymphocytes are regenerated every 3–4 days then, in the labelled thymus-grafted mice 3–6 days after labelling, up to 1250 mg. of labelled cells must either have died or migrated from the thymus tissue to make way for the next generation of small lymphocytes. From the data, calculations showed that fewer than 0·5 per cent of thymic cells (from host thymus or grafts) could have seeded in the host lymphoid tissues between days 3 and 6 after labelling. Since labelled lymphocytes were not demonstrated in excessive numbers in the liver, kidney or gut of these thymus-grafted animals, the results imply that very few lymphocytes produced in the host thymus or thymus grafts ever left these tissues.

Perhaps animals carrying multiple thymus grafts have some special mechanism for preventing the escape of these excess lymphocytes from the grafts. However, the labelling patterns in thymus grafts were identical with those in the normal thymus and, since no excess of pyknotic cells was observed in such thymus grafts, this hypothesis requires the existence of a mechanism which gives little visible evidence of its existence but which destroys small lymphocytes in the thymus every 3–4 days. We feel that such a mechanism for cell destruction does exist and that this same mechanism operates in the normal thymus.

It seems likely that relatively few cells produced in the thymus ever leave the organ, the vast majority being destroyed on a non-random basis at the end of 3–4 days. This leaves as a major unsolved problem the purpose of most of the intense lymphopoiesis occurring in the thymus.

Although few thymic cells leave the organ it should not be concluded that such limited cell migration as does occur is necessarily of little importance to the body. It is quite clear from Fig. 3 and direct labelling experiments in the rat (Little *et al.*, 1962) that the average life-span of non-thymic lymphocytes is much longer than that of thymic lymphocytes (possibly 30–100 days compared with 3 days). Even a small percentage of cells migrating from the thymus could be a valuable source of new cells for the non-thymic lymphoid tissues and, on this basis alone, thymectomy could be expected to lead to a slow decline in non-thymic lymphoid organ weights.

Certain data appear to contradict the conclusion that few thymic lymphoid cells leave the organ. A number of investigators have shown in chromosome-marker experiments that thymus-grafted neonatally thymectomized mice contain many mitotic cells of graft origin in host lymph nodes and spleen (Harris and Ford, 1964; Leuchars *et al.*, 1964). These observations have not established the time at which this cell migration from the graft occurs. The grafted thymus is commonly mechanically disrupted during the grafting process, substantial necrosis of thymus tissue occurs immediately after grafting and this is accompanied by a widespread breakdown of the architectural framework of the tissue (Metcalf and Wakonig-Vaartaja, 1964). During the immediate post-grafting period it is possible that many cells may escape from the disrupted graft tissue, lodge in host organs and subsequently proliferate freely—particularly if the host is depleted of lymphocytes. This cell migration may no longer occur once the architecture of the thymus grafts is restored. The evidence from the injection of [^{3}H]thymidine into multiple-

thymus-grafted mice just described comes from animals in which the architecture of the thymus graft was fully restored before tests were applied for cell migration. In this situation (more nearly related to the functional state of the normal thymus) no large scale migration was demonstrated.

Similar experiments using [^{3}H]thymidine-labelled, multiple-thymus-grafted mice suggest that weight loss in the thymus following the injection of cortisone is not due to cell migration from the thymus but to the accelerated destruction of thymic lymphocytes *in situ* (Metcalf and Matsuyama, 1965). It seems unlikely, therefore, that during the stress of infections or the mild stress of antigen administration there is any substantial excess migration of potentially useful lymphoid cells from the thymus to locally-reacting lymphoid organs.

LYMPHOPOIESIS IN THYMIC LYMPHOMA TISSUE

More than 90 per cent of AKR mice spontaneously develop lymphoid leukaemia and in most cases the disease begins in the thymus which is progressively replaced and enlarged by leukaemic (lymphoma) cells to form a massive thymic lymphoma. Certain obvious differences exist between the normal and the neoplastic thymus: first, the total population size of thymic lymphoma tissue increases progressively—under optimal conditions doubling approximately every 24 hours—whereas the population size in the normal thymus remains constant over long periods; and second, the percentage of primitive cells in AKR lymphoma tissue (mean values: 25 per cent large lymphoma cells, 56 per cent medium lymphoma cells), although highly variable from one animal to another, is always greatly in excess of the percentage in the normal AKR thymus (mean values: 1 per cent large lymphocytes, 10 per cent medium lymphocytes) (see Figs. 1 and 2). Previous studies indicated that mitotic indices in primitive lymphoma cells were lower than in normal primitive

thymic cells (Nakamura and Metcalf, 1961). Recent radioautographic data obtained in our laboratory have revealed in more detail the reasons for the slower division rate of lymphoma cells (Metcalf and Wiadrowski, 1966). Large lymphoma cells divide almost as rapidly as normal large lymphocytes, but medium lymphoma cells, due to a prolonged presynthetic (G_1) period, divide at less than half the rate of normal medium lymphocytes (Table I). Since medium lymphoma cells are the predominant cell type in lymphoma tissue this accounts for the overall lower mitotic indices of lymphoma cells.

The slower proliferative rates of lymphoma cells compared with their normal equivalents is in agreement with comparative studies in man which indicated that leukaemic blast cells divide more slowly than normal bone-marrow blast cells (Killman *et al.*, 1963; Mauer and Fisher, 1963; Gavosto *et al.*, 1964).

Although small lymphoma cells are an integral part of the tumour population, it is of some interest that, like normal small lymphocytes, small lymphoma cells are predominantly non-dividing cells.

As with normal thymus cells, reutilization of [^{3}H]thymidine prevents accurate analysis of the division patterns of primitive lymphoma cells but, in general terms, large lymphoma cells divide asymmetrically to form both large and medium lymphoma cells. Similarly, medium lymphoma cells divide to form both medium and small lymphoma cells. The percentage distribution of large, medium and small lymphoma cells remains reasonably constant in the enlarging tumour population (Metcalf, Nakamura and Wiadrowski, 1965). This means that a larger fraction of the rapidly-dividing, large lymphoma cells must divide to form medium lymphoma cells and a smaller fraction of the slowly-dividing, medium lymphoma cells must divide to become small lymphoma cells. In both cell types, however, more than 50 per cent of the offspring must remain parental in type as this provides the basis for the expanding size of the lymphoma

population. The differences between the percentages of parental-type offspring in neoplastic and normal populations may be of the order of 70 per cent for lymphoma cells and 45 per cent for normal cells.

No correlation was found in individual thymic lymphomas between the degree of differentiation in these tumours and the rates of division of the lymphoma cells. These two characteristics appear to vary independently of one another, but both are abnormal in all lymphomas.

REGULATION OF NORMAL THYMIC LYMPHOPOIESIS

The thymus may best be regarded as an epithelial and reticulum-cell sponge which is capable of providing a suitable seeding out tissue for circulating potential lymphoid stem cells; these cells proliferate and differentiate to form a mass of lymphoid cells distending the interstices of the thymic sponge. The size of the thymus is limited by the carrying capacity (size) of the thymus sponge. Half a neonatal thymus, when grafted, achieves a maximum weight exactly half that of a whole neonatal thymus graft, and a strictly linear relationship exists between the number of glands (that is, the amount of spongework) grafted and the total weight of thymus graft tissue resulting (Table III). Further, partial thymectomy is not followed by regeneration of the part remaining (Metcalf, 1964*a*). When the spongework of the thymus is made to collapse inwards by destruction of the lymphoid cells in the interstices, as after cortisone administration, prompt regeneration of the organ occurs (Ishidate and Metcalf, 1963).

Some appreciation of the factors regulating thymic lymphopoiesis has been gained from an analysis of the reasons for the decrease in the size and mitotic activity of the thymus with increasing age. When multiple neonatal thymuses were grafted into very old C57BL mice some decrease was seen in the size attained by these grafts compared with similar grafts in young

hosts (Table III). However, in these old animals the total weight of thymus-graft tissue achieved far exceeded the maximum weight of the normal C57BL thymus (60 mg.) in young adult life. Since all thymus grafts are repopulated by host cells it appears that a limitation of the supply of these circulating potential stem cells is not a significant limiting factor for thymus size in old age. Similarly, the fact that young thymus grafts in very old animals grew vigorously and exhibited high mitotic activity

TABLE III

THYMUS-GRAFT WEIGHTS ATTAINED BY MULTIPLE WHOLE C57BL THYMUS GRAFTS IN YOUNG AND OLD C57BL MICE†

Age of recipient at grafting (months)	*Number of thymus glands grafted per recipient*	*Number of recipients*	*Mean host thymus weight (mg.)*	*Mean weight of individual thymus grafts (mg.)*	*Total thymus-graft weight per recipient (mg.)*
2	1	28	47	24	24
	6	10	45	19	113
	12	41	42	22	262
	24	2	48	25	609
	48	2	36	24	1160
20	12	18	16	16	185
	24	2	15	19	467

† All grafts were from 1-day-old donors and were placed in the subcutaneous tissues. Mean weight of each donor thymus gland = 3 mg. Grafts were weighed 4 weeks after grafting.

indicates that "intracellular ageing" in the repopulating lymphoid stem cells, or some general hormonal change inimical for active lymphopoiesis, are not factors in determining the decline in proliferative activity of thymic lymphoid cells in ageing animals.

In a previous study (Metcalf *et al.*, 1961) the effects of ageing on the size of single C3H thymus grafts were determined in post-involutional hosts. The results are shown graphically in Fig. 5: the weight of the grafts increased to a maximum, then fell, and the timing of this involution with age of the grafts coincided exactly with the timing of involution in the normal C3H thymus. This experiment indicates that age changes in thymus-graft

weight are due to changes inside the organ itself—specifically to ageing of the surviving epithelial and reticulum-cell elements of the graft. Other factors can and do affect thymus weight, for example adrenalectomy, gonadectomy and the repeated injection of ACTH, cortisone, oestrogens and testosterone. None of these factors actually affects the *timing* of the involution that occurs with age, but merely the absolute weight changes of the thymus (Dougherty, 1952).

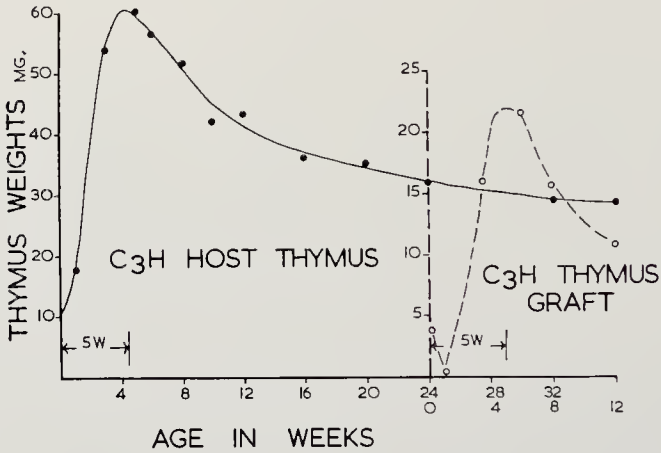

FIG. 5. Weight changes in neonatal subcutaneous C3H thymus grafts in 6-month-old (postinvolutional) C3H hosts. Involution occurs in thymus grafts at the same age as in normal C3H mice. Each point represents mean values for 30 thymus grafts.

Other, similar grafting experiments suggest that the epithelial and reticulum-cell elements of the thymus are also responsible for determining strain and sex differences in thymic size and mitotic activity (Metcalf *et al.*, 1961).

Does the thymus epithelial and reticulum-cell complex actually stimulate lymphoid cells to divide or merely allow the expression of these cells' inherent capacity to divide? To some degree this is a question of semantics since it is likely that "proliferative stimulation" for all cells in the body merely represents varying degrees

of release from the proliferative inhibition imposed by differentiation and tissue interaction.

It can be argued that, if the lymphocytes in the lymph nodes need antigenic stimulation to exhibit maximum mitotic activity, some comparable intrinsic proliferative stimulus must be present in the thymus, for mitotic activity of the thymic lymphocytes, although equally high, does not appear to be dependent on antigenic stimulation.

That a true positive stimulus for proliferation is exerted by the non-lymphoid cells of the thymus is suggested by graft experiments involving parental to F_1 grafts and young grafts in old hosts (Metcalf *et al.*, 1961). Here the observed quantitative differences in rates of lymphoid proliferation could only have been due to differences in a positive proliferative stimulus exerted by the surviving epithelial and reticulum-cell elements of the grafts.

The anatomical localization of mitotic activity in the thymus cortex provides some clues to the mechanisms involved in the regulation of lymphopoiesis. This subject has been discussed in full elsewhere (Metcalf, 1964*b*) but, briefly, it may be said that mitotic activity tends to be concentrated in the peripheral parts of the cortex adjacent to the capsule and along the radial vessels with their sheaths of reticular-epithelial cells.

Lymphocyte mitoses were also concentrated around reticulum cells whose granules stained specifically with the periodic acid-Schiff stain (PAS cells). PAS cells are concentrated in the outer third of the cortex of the thymus—the region exhibiting the highest degree of mitotic activity (Metcalf and Ishidate, 1962). The association of PAS cells with mitotic lymphoid cells is most evident in thymuses exhibiting high mitotic activity, for example, the AKR thymus and the regenerating thymus. It is of some interest that PAS cells are phagocytic cells and that a similar PAS-positive material appears to be secreted by the non-phagocytic epithelial cells of the thymus.

Graft experiments using fragments of thymus tissue have indicated that only fragments containing medullary epithelial cells can give rise to grafts containing typical cortical lymphoid tissue (Metcalf, 1963). This suggests that medullary epithelial tissue is necessary in some way for sustaining cortical lymphopoiesis.

At least three cell types (cortical epithelial cells, PAS cells and medullary epithelial cells) appear to be related to the maintenance of lymphopoiesis in the thymic cortex. The simplest concept of their interaction is that together they provide both a suitable microenvironment for the seeding out of potential lymphoid stem cells and the actual proliferative stimulus for these cells. The system involved may well be an analogue of the system involved in the regulation of erythropoiesis. A thymic humoral factor of epithelial-cell origin (cf. erythropoietin of renal origin) may stimulate stem cells to proliferate but only if these stem cells are in a suitable microenvironment, conditioned by the cortical meshwork of reticular-epithelial and PAS cells (cf. the special cellular stroma of the bone marrow and spleen which allows erythropoietin to act on potential erythropoietic stem cells in these locations but nowhere else in the body).

REGULATION OF LYMPHOPOIESIS IN THYMIC LYMPHOMA TISSUE

At first sight lymphoma tissue appears to be fully autonomous with no significant stimulation or regulation by the host, for the size of the population of lymphoma cells increases unchecked until the death of the animal supervenes. Furthermore, in individual animals, thymic lymphoma tissue and lymph node lymphoma tissue show identical percentages of primitive cells and identical (subnormal) mitotic activity (Metcalf, Nakamura and Wiadrowski, 1965). This suggests that thymic lymphoma cells are no longer under the influence of, or are no longer responsive to, the stimulation of the normal thymic epithelial and

reticulum cells which still survive in the thymic lymphoma mass. No tests have been made to determine whether or not proliferative activity of the lymphoma cells is stimulated by the injection of antigens, although such information would be of value.

It is evident that lymphoma cells have escaped from the normal regulating mechanisms, for these cells will proliferate progressively when transplanted to normal young adult mice: but is this escape complete? Lymphoma cells do differentiate in a superficially normal manner, producing ultimately what appear to be non-dividing end cells (Metcalf and Wiadrowski, 1966). Any degree of differentiation implies some responsiveness to host control mechanisms since differentiation is not a feature of cells exhibiting continuous growth under *in vitro* conditions.

More attention should be paid to determining the extent to which lymphoma cells are still responsive to control mechanisms, and to determining the nature of these mechanisms. In view of the suspected biological interaction between reticulum cells and lymphocytes, attention should also be paid to the behaviour and function of the large phagocytic cells which are a characteristic component of all lymphoma tissues.

SUMMARY

Thymic lymphopoiesis has been measured by direct mitotic counts and tritiated-thymidine uptake studies in both the normal and the neoplastic mouse thymus. Although division of lymphoid cells in the thymus is very rapid (large lymphocytes 6·8 hours, medium lymphocytes 8·2 hours) it may be no more rapid than the rate of division in lymph nodes under conditions of maximal antigenic stimulation. Most small lymphoid cells produced in the thymus do not appear to leave the organ but die, on a non-random basis, in the thymus after 3–4 days.

Thymic lymphopoiesis is regulated by the epithelial and reticulum-cell elements of the thymus and does not appear to be responsive to antigenic stimulation.

Thymic lymphoma cells divide more slowly than normal thymic lymphoid cells and appear capable of a variable degree of differentiation. The mechanisms regulating this differentiation are unknown. Lymphoma-cell populations increase in size exponentially because of the high proportion of parental-type offspring resulting from the divisions of primitive lymphoma cells.

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GENERAL DISCUSSION

Clark: It is difficult to explain what happens to the tritium-labelled small lymphocytes after three days' residence in the thymus. Dr. Metcalf has suggested that most of them actually die there after this time but if this is so it is surprising that there is so little detectable reutilization of thymidine in the thymus.

Metcalf: One does see pyknotic (dying) lymphoid cells of all ages in the thymus but the numbers are not large (Metcalf, D. [1964]. *In* The Thymus in Immunobiology, p. 150, ed. Good, R. A., and Gabrielsen, A. E. New York: Hoeber). I think that this is because most dying lymphocytes disintegrate rather than become pyknotic. What is more important is that one *does* find extensive reutilization of tritiated thymidine in the thymus. In radioautographs the label can be followed from day to day as it moves from the large cells to the small lymphocytes and back to the large cells again (reutilization). This reutilization is so extensive that it makes interpretation of the patterns of cellular division in the thymus practically impossible.

Clark: Dr. Ford has presented evidence that thymic lymphocytes do leave the thymus and may migrate to the lymph nodes. Therefore the failure of Dr. Metcalf's multiple thymic grafts to populate the peripheral lymph nodes with thymic lymphocytes cannot to due to failure of lymphocytes to leave the thymus: we must try to find another explanation. Could there be a mechanism which controls the number of lymphocytes that occupy peripheral lymph nodes? Might this act at the level of the post-capillary venules which, as Professor Gowans has shown, is the site where the lymphocytes leave the blood to populate the lymph nodes? Dr. Ford, do you think there might be a homoeostatic mechanism at this level?

Ford: The cytological evidence for a flow of lymphocytes from the thymus to lymphoid tissue elsewhere is derived from an artificial situation in which the thymus is grafted on to neonatally thymectomized hosts, as originally shown by Dr. Miller. Because our experiments are less physiological than, say, Dr. Metcalf's, I have not stressed the process of outward flow of lymphocytes from the thymus although I believe that the evidence favours this. There may be a regulatory mechanism for the flow, but it is difficult to plan an experiment in which this could be demonstrated. In parabiotic mice in which the thymus of one mouse only has been removed, we have found a greater proportion of cells from the intact animal in the lymph nodes of its thymectomized partner rather than the other way round. I am not going to stress this observation as it could be interpreted in several different ways. My colleague Dr. Barnes and I think that these results favour a cellular interpretation, that is, they reflect an outward flow of lymphocytes from the one remaining thymus, but it is not possible to draw a decisive conclusion from this work. I do not think that our data and Dr. Metcalf's are incompatible.

Metcalf: I agree with Drs. Ford and Clark that there may be a mechanism which regulates the number of lymphocytes that migrate to peripheral lymphoid tissue from massive thymus grafts. The point I wanted to make was that, since lymphopoiesis is absolutely identical in thymus graft tissue and in the normal thymus, this mechanism must operate in both these tissues; when the body does not need lymphocytes from the thymus it has to have an effective mechanism for destroying them. Dr. Ford's work with thymus grafts in neonatally thymectomized animals has shown the presence of lymphoid cells of graft origin in the host lymph nodes (Harris, J. E., and Ford, C. E. [1964]. *Nature, Lond.*, **201**, 884; Miller, J. F. A. P., this volume, p. 166). The difference between these results and our own does not come from using thymectomized rather than normal recipients for the grafts. When a neonatal thymus is handled the capsule breaks immediately

and a flood of milky, cell-containing fluid spreads out from it. The thymus is invariably ruptured at the time of grafting and there is also anoxic death and disruption of architecture within the gland in the first few days after grafting. It would therefore be easy, for at least a week after grafting, for thymic lymphocytes to leave the local area of the graft. If the host animal is in a lymphocyte-depleted state these cells will probably seed in host lymphoid organs. But these findings do not prove that the flow of lymphocytes from thymus grafts to the host is a continuing process. In our experiments we used animals in which grafts had been established for a month and in this situation we found little evidence of outflow of cells from the thymus. Nevertheless, even if only 1 per cent of thymus or thymus-graft lymphocytes do migrate to the non-thymic lymphoid organs this is immensely important. If these cells are of a type that can respond to antigens they can quickly undergo clonal proliferation and become a major fraction of the lymphocyte population of the lymph node. What I do not understand is the reason for the other 99 per cent of lymphopoiesis in the thymus. This seems an extraordinarily wasteful process.

Nossal: This upper limit figure of 1 per cent, derived from indirect calculations, agrees well with what we thought was the approximate proportion of thymic small lymphocytes leaving the thymus in some experiments of mine (Nossal, G. J. V. [1964]. *Ann. N.Y. Acad. Sci.*, **120**, 171). We counted the number of cells leaving the thymus of the guinea-pig after direct labelling with tritiated thymidine and when thymidine was excluded from the rest of the body. It is difficult to make accurate calculations but, in the adult animal, of the order of 1 per cent of the total labelled pool of small lymphocytes in the thymus had seeded out peripherally. This is an extraordinarily low figure. We have also found that in the newborn guinea-pig—a much more mature animal at birth than the mouse—this seeding out was increased by a factor of at least five. Dr. Metcalf, have you done any work with newborn hosts as well as with newborn thymus grafts?

Metcalf: No, but I think that the results of such work would coincide with Dr. Ford's findings. The only effective way to label a graft before transplanting it is by the use of chromosome markers. Unfortunately with this technique you cannot say if cellular migration is a continuing process or a single event.

Nossal: You misunderstood my question; I wanted to know if you had done multiple thymus grafting in the newborn mouse.

Metcalf: With our technique, unfortunately, we have to wait one month before the thymus grafts are fully reconstituted and can be tested. By this time we have a one-month-old, young-adult mouse, not a neonatal animal.

Kaplan: One of my students, Dr. Irving Weissman, when he was working in Professor Gowans' laboratory last year, did some experiments which are relevant to this discussion (unpublished data). Weissman gave tritiated thymidine by continuous infusion into the thymus of the rat at various ages. At the same time he gave large doses of non-radioactive thymidine to the rest of the animal to eliminate any possibility of the label appearing elsewhere because of non-specific leakage. This work demonstrated quite convincingly that seeding of labelled cells from the thymus to the lymph nodes and the spleen does occur. Weissman also found, as Dr. Nossal has just said, that there is a higher rate of seeding in the very young animal.

Burnet: Professor Gowans, has anyone measured the percentage of labelled cells that can be accounted for in lymphoid tissues other than the thymus?

Gowans: I am not quite sure how such calculations should be done. Dr. Nossal, how did you arrive at your figure of 1 per cent for the number of cells leaving the thymus?

Nossal: We used the method described by Dr. Metcalf. We gauged the weight of the different lymphoid organs, arrived at an approximate idea of the total weight of these and, with the knowledge of the known mitotic rates in the various organs, we were able to compute approximately the outflow of lymphocytes

from the thymus to the peripheral lymphoid tissue. We presumed that there was a random distribution of these cells throughout the lymphoid tissue.

Gowans: Your figure refers to 1 per cent of what?

Nossal: One per cent of the cells in the thymus.

Gowans: I am still not clear how you did the calculation.

Nossal: We calculated the total number of cells that had been generated in the thymus in a given time and then tried to track down that number. The number of migrants that we found could have accounted for approximately 1 per cent of the total number of lymphocytes in the thymus, that is, for 1 per cent of 10^6 or 10^7 cells.

Mackay: If there were phasic changes in the outflow of cells from the thymus this would fit with Dr. Clark's ideas about regulatory mechanisms. In other words, if we could visualize the thymus as a dam from which cellular outflow is regulated according to need, we might have an explanation for the constant high rate of cellular proliferation.

Metcalf: Lymphocytes in sections of the thymus appear to be smaller than lymphocytes in the lymph nodes and this has always been thought to be due to tighter packing of cells in the thymus. We have measured these cells in the Coulter counter and there is no doubt that thymic lymphocytes really are smaller than lymph node lymphocytes (Fig. 1). The small lymphocytes in the lymph nodes are also more heterogeneous than the thymic small lymphocytes. If there is a large scale migration of cells from thymus to lymph nodes then the lymphocytes must also increase in size and heterogeneity very rapidly as they migrate peripherally, and this seems unlikely.

An experimental model in which the host reservoir of lymphocytes is depleted and yet intact thymus tissue is present can be represented by a thymus graft in a long skin pedicle. In such a model the host animal can be irradiated whilst the graft in the skin pedicle is shielded.

Dameshek: Is it the small lymphocytes that migrate to the periphery or could it be the large lymphocytes? I think there are some problems about terminology here. The cell which Dr. Metcalf has called a thymic large lymphocyte looks like what I would call a blast cell or immunoblast. (Dameshek, W. [1963].

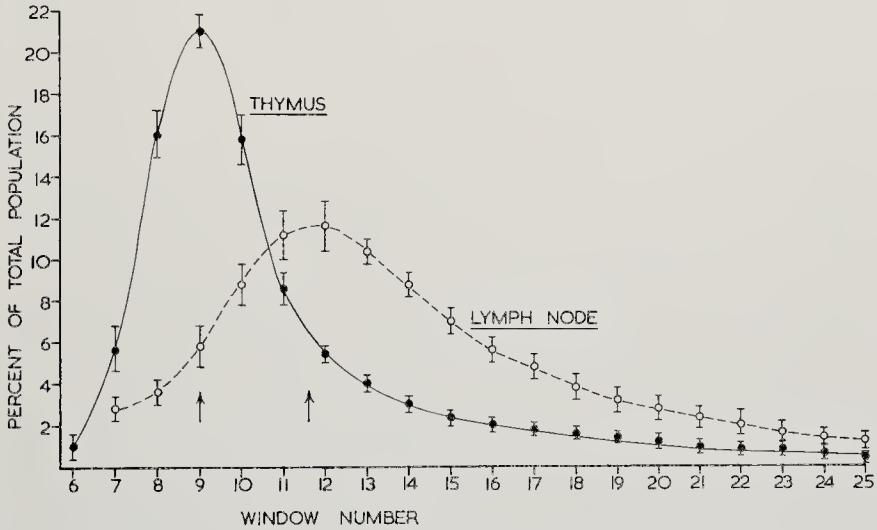

FIG. 1 (Metcalf). Coulter-counter analysis of cell suspensions from thymus and subcutaneous lymph nodes of 2-month-old AKR mice, 6 mice in each group. Vertical bars represent standard deviations of group means. Note the difference in size of small lymphocytes (large peaks) from the two tissues.

Blood, **21**, 243). This is a primitive-looking cell with prominent nucleoli and according to Le Blond's classification these cells are not large lymphocytes. Experiments with hamsters (Adner, M. M., Sherman, J. D., and Dameshek, W. [1965]. *Blood*, **25**, 511) have shown that the percentage of primitive cells in these mammals remains high for about two weeks after birth, and that runting after thymectomy develops only in animals that are thymectomized up to two weeks of age. This is in contrast to the mouse in which runting appears only if the mouse is thym-

ectomized directly after birth. The cell that migrates to the periphery, therefore, may not be a small lymphocyte but a much more primitive cell, such as a blast cell. Dr. Metcalf, what do you think about this?

Metcalf: The "large lymphocytes" we refer to have a total cell diameter, in cell smears, greater than 11 μ and the "medium lymphocytes" have a cell diameter of 7-11 μ. This terminology is the one most commonly used by workers who handle mouse and rat thymic tissue.

The large lymphocyte in the blood corresponds in size with the medium lymphocyte in the tissues. In our studies on the migration of cells from the thymus we have investigated the possibility that primitive cells rather than small lymphocytes are migrating from the thymus, but we have found no change in the percentage of primitive cells or their labelling pattern in the lymph nodes or blood in multiple-thymus-grafted animals. I agree that in the neonatal thymus there is a far higher percentage of large and medium cells—up to 30 per cent in the mouse—than in the adult thymus. However, in the mouse, this percentage falls quite rapidly during the first week of life. (Axelrad, A. A., and Van Der Gaag, H. C. [1962]. *J. Natn. Cancer Inst.*, **28**, 1065).

The cytologies of lymph node and thymus in the mouse are very different. The large lymphocyte in the lymph node is a tremendous cell which really does justify the name of blast cell. There is a disturbing discontinuity in the spectrum of cell sizes in the lymph nodes. There are very large blast cells, but the cells next in size to them—the medium and then the small lymphocytes—are much smaller than the large cells (although they are larger than equivalent cells in the thymus). In the thymus, in contrast, there is a perfect gradation in cell size from large lymphocytes, to medium, to small. There is evidence that most blast cells in the lymph node may not be concerned with generating lymphocytes, for it has been shown that the blast cells in the germinal centre

are probably not the actual producers of the lymphoid cells in the surrounding follicle (Fliedner, T. M., *et al.* [1964]. *Ann. N.Y. Acad. Sci.*, **113**, 578). In the thymus it is easy to follow the passage of radioactive label through the descending size spectrum of thymic lymphocytes. This is more difficult for cells in the lymph node and I am not really convinced that the blast cells are part of the lymphoid series at all.

Burnet: Does the evidence from relabelling of the lymphocytes in the thymus suggest that much of the material from the missing lymphocytes is incorporated in the new cells which are being produced continuously?

Metcalf: Yes. This passage of label from small lymphocyte back to primitive lymphocyte is clearly seen in Table II in my presentation (this volume, p. 250). Three days after initial labelling the content of label in large and medium lymphocytes starts to rise and the most obvious source of this additional label is the adjacent small lymphocytes. Cell-turnover data have suggested that these small lymphocytes must be beginning to disintegrate at about this time. We do not know what fraction of the labelled breakdown products is trapped and saved but, judging from the relatively rapid fall in total-organ grain count which starts on the third day after the initial labelling, it cannot be 100 per cent. It is possible that only about 30 per cent of the label is reutilized in the thymus, although some may be reutilized by other cells in the body.

A statement often quoted in the literature concerns the effect of cortisone on the migration of lymphocytes from the thymus. It is said that the thymus decreases in size after the administration of cortisone because thymic lymphocytes migrate out of the gland. We have checked this proposition with labelled cells in multiple-thymus-grafted animals and we have found absolutely no evidence for it. These cells do not migrate out of the thymus, they are simply destroyed locally as the thymus volume decreases. (Matsuyama, M., and Metcalf, D. Unpublished data.)

Good: We might note here the tremendous accumulation of small lymphocytes in the peripheral blood in infections due to *Haemophilus pertussis.* Morse, at the Rockefeller University,† (Morse, S. I. [1965]. *J. exp. Med.*, **121**, 49) has presented evidence that there is a depletion of small lymphocytes in the thymus and all other lymphoid tissues after the injection of pertussis antigen in mice. Morse suggests that lymphocytes from the lymphoid tissues are dumped into the peripheral blood and that this explains the increased blood lymphocyte count. If this is right, and the increase in lymphocytes in the blood in these circumstances is due not to an absolute increase in the number of lymphocytes in the body but to an increased release of lymphocytes from the tissues where these cells are stored, this model might provide another useful approach for future work on animals with local thymus grafts. Morse's observations may have revealed a fundamental reflex relating to the delivery of the small lymphocyte. We have looked at this in some very primitive animals, for example the lampreys (*Petromyzon marinus*), in which the development of small lymphocytes is just starting to evolve and we have seen the same changes as Morse showed in the mouse. Whatever the evolutionary adaptation that gave rise to the development of this response to *Haemophilus pertussis* and the advantage for survival derived from it, we may postulate that it is at least four hundred million years old.

Dameshek: In patients with chronic lymphocytic leukaemia treated with cortisone there is a striking reduction in lymphoid masses and, simultaneously, a great temporary increase in the blood lymphocytes.

Irvine: Dr. Metcalf, is there any evidence for or against the loss of unwanted lymphocytes through the alimentary tract? It is important to know about this before concluding that the majority of lymphocytes that originate in the thymus are actually destroyed there.

† Formerly the Rockefeller Institute.

Metcalf: It is very difficult to measure the number of lymphocytes that are passing through the wall of the intestines. The only evidence that I have about this comes from radioautographic studies of sections of the intestinal wall. We examined these blind and could not distinguish which sections were from the grafted animals and which from the controls. We could detect no difference in the number of labelled cells in this area in the two groups. But what I wanted to emphasize was that we could not find these labelled lymphoid cells in the gut, or the peripheral lymphoid tissues, or anywhere else either.

Furth: I am intrigued by the fate of the 99 per cent of lymphocytes that apparently disappear. B. J. Bryant (1963, *J. Cell Biol.*, **18,** 515) has shown that, after breakdown, DNA from the lymphocytes can be incorporated into other cells. His work suggests that although most metabolites are excreted nuclear fragments may be utilized in the reconstruction of nuclei that are needed elsewhere; for example, this applies to the intestinal epithelial cells which, like the lymphocytes, are steadily lost. Experiments with radioactive isotopes to confirm these findings and to try to work out a balance for the fate of labelled DNA from disintegrating lymphocytes may disclose how much of it appears in the urine and faeces and in what form, and how much is incorporated in the nuclei of new lymphocytes and other new cells.

Metcalf: I agree. The multiple-thymus-grafted mouse would be a most suitable experimental animal for this sort of study. It is possible to have almost unlimited amounts of thymus tissue in this animal so that the metabolic products of thymic cell breakdown, and their reutilization, can readily be traced. In preliminary studies in multiple-thymus-grafted mice we have tried to discover if labelled thymic lymphoid breakdown products are incorporated in the regenerating liver. We have not yet looked at bowel epithelial cells for evidence of reutilization of radioactive label originating in the thymus graft.

Kaplan: I agree with Dr. Metcalf that to explain the mystery of the disappearance of lymphocytes from the thymus one need not invoke reutilization of thymidine in the thymus as a necessary consequence of the destruction of the cells. The destruction of small lymphocytes in the thymus after the administration of corticosteroids or following irradiation is a comparable situation. In these circumstances the small lymphocytes die extremely rapidly—there is a tremendous decimation of the lymphocyte population of the thymus in the first three hours after a test dose of radiation or corticosteroid. A massive amount of nuclear debris from the small lymphocytes next appears in the macrophages of the cortex of the thymus but, twenty-four hours later, both macrophages and nuclear debris have disappeared. We do not need to assume that the three to four-day-old lymphocytes are destined to be removed synchronously: their disappearance is more likely to be randomly distributed. Because the process of death and destruction of the small lymphocyte is very rapid—it may take only a half-hour—at any moment in time there will be only 2 per cent of this dying population of cells visible in a section of the thymus and this small proportion might easily be overlooked.

Testosterone and other androgens are also powerful thymolytic agents but, unlike the corticosteroids, they do not cause involution of the peripheral lymphoid system: their action is specific for the thymus. They might, therefore, be used to study the kinetics of the cell system in which the thymus is selectively depleted of lymphocytes while the peripheral lymphoid system is maintained.

Another paradox is the disappearance of donor-type cells from thymus grafts. Dr. Metcalf, can you explain why the donor cells should not compete on reasonably favourable terms with the host cells? As there is no immunological incompatibility, it is puzzling that the thymus graft can provide a micro-environment in which the host cells can proliferate indefinitely but the donor cells cannot.

Metcalf: At most ages in the mouse, the number of pyknotic cells present in the thymus is about the same as the number of mitotic cells. (Metcalf, D. [1964]. *In* The Thymus in Immunobiology, p. 150, ed. Good, R. A., and Gabrielsen, A. E. New York: Hoeber). Most people believe that cell death (through pyknosis) is a slow process and takes much longer than 30-40 minutes—the time taken for one mitotic cell division. It is thought, therefore, that the number of cells dying in the thymus is far less than the number being produced there.

I would like to emphasize what Dr. Kaplan has just said, namely, that lymphocyte destruction can be a very rapid process. In our radioautographic studies on lymphoma tissue we have often observed labelled pyknotic lymphocytes in phagocytic cells one hour after labelling. Such cells were obviously in DNA synthesis at the time of labelling, but within one hour they were pyknotic remnants within phagocytic cells, and frequently the phagocytosed cells had had most of their stainable DNA broken down; all this within one hour of being in active DNA synthesis!

We have confirmed Dr. Kaplan's finding that testosterone has a selective cytolytic effect on the thymus. Thymus grafts, however, are less sensitive than the normal thymus to cortisone, and also to pregnancy and lactation. In their relative insensitivity to these agents, thymus grafts resemble lymph node tissue (Metcalf, D. [1964]. *Transplantation*, **2**, 541). O. Stutman and S. B. Zingale (*Proc. Soc. exp. Biol. Med.* [1964]. **117**, 389) claimed that thymus grafts, also, behave like lymph nodes and develop germinal centres and plasma cells when antigenically stimulated, but we have not been able to confirm this.

The disappearance of the donor cells in thymus grafts may be related to the possibility that haemopoietic cells are only capable of a finite number of divisions (Siminovitch, L., McCulloch, E. A., and Till, J. E. [1964]. *J. cell. comp. Physiol.*, **64**, 23). In thymus grafts large and medium lymphocytes divide three to four times per day. If the original donor cells persist in the grafts for an

average of 20 days the cells might have to divide 60 to 80 times. McCulloch and Till's work (1964, *loc. cit.*) suggests that the colony-forming cells are capable of dividing about 50 to 100 times. The eventual disappearance of mitotic donor cells from thymus graft populations could be related to the limited number of cell divisions which haemopoietic cells can undertake.

Burnet: I agree.

Auerbach: Dr. E. Chaperon, a student of the late Harold Wolfe, has been studying the role of the thymus in chickens in our laboratories. The results of two of his experiments are not in line with current concepts on the relationship of the thymus and the bursa to normal homotransplantations and to antibody formation in response to soluble antigens. Dr. Chaperon (unpublished material) gave a group of chickens a massive dose of bovine serum albumen (BSA) at hatching, and then removed the thymus; this is the chicken parallel for Talmage's experiments on mice (Claman, H. N., and Talmage, D. W. [1963]. *Science*, **141**, 1193). Chaperon next studied the recovery from unresponsiveness to BSA which normally occurs in such animals after about 12 weeks and he found that thymectomy had drastically increased the length of this recovery period. In some of the birds recovery never occurred and in others the final rate of antibody formation was very much reduced. This certainly suggests that the thymus is concerned in the production of antibodies against BSA.

Dr. Chaperon thymectomized chickens at hatching, and, about eight weeks later, he irradiated them with a sublethal dose of X-irradiation—900 r. He then studied the recovery of these chicks' ability to make antibody by the method described by M. D. Cooper, R. D. A. Peterson and R. A. Good (1965. *Nature, Lond.*, **205**, 143). Chaperon found, like Professor Good and his colleagues, that antibody production against BSA did not regenerate at all. Ten of the birds that had been thymectomized and irradiated and were unresponsive to BSA were grafted with both skin autografts and homografts, and in all these ten birds the

homografts were rejected at the "normal" time. There was thus no difference in the persistence of skin grafts between the thymectomized irradiated birds and normal birds, even though the birds in the former group could not make good antibodies against BSA. After the ten unresponsive birds had rejected the skin grafts they were tested with human γ-globulin and did not make the appropriate antibodies to this antigen. These two series of experiments show that the thymus is involved in the recovery of the reactions to soluble antigens but that apparently it has little effect on homotransplantations. I do not know how to explain these two incongruous sets of results, but they should be considered with the ones reported from Professor Good's department (Cooper, Peterson and Good, 1965, *loc. cit.*).

Good: The first set of experiments you have described are not at all out of phase with current concepts. The interaction of both thymic and bursal systems is needed to effect a full immune response involving antibody production. Chaperon's observations have shown that the response to certain antigens in the chicken is similar to the response in the mouse: that is, absence of the thymic system reduces antibody-producing capacity. Immunological tolerance and memory, however, may not be functions of the specialized immunoglobulin-producing system but of the bursal system. We interpret our own observations to mean that a full immune response with antibody production must occur, either through the macrophage system or the thymic system or both, into the production system.

The results of the homograft experiments are rather difficult, but not impossible, to explain. In chickens irradiated in adult life, when at least some of their thymic system is intact, homograft rejection may occur even if the response to circulating antibodies is impaired. Our evidence indicates that thymic and bursal systems in the chicken seem to be completely separate, although interacting, so I do not find these results too hard to digest.

Burnet: We have heard that the thymus may have more than

one hormonal effect and it has been suggested that the bursa also has a hormonal function. If we accept the general clonal theory on the emergence of lymphoid cells from the thymus, it seems possible that one or more thymic hormones are needed to maintain the viability of these cells. But there may also be another hormone related, perhaps, to the lymphoid tissue of the gut (including the bursa) which is necessary if the lymphocytes are to give rise to antibody-producing plasma cells. This fits in well with Professor Good's approach. There is, too, some experimental evidence which suggests that bursal tissue in a diffusion chamber has a regenerating effect on antibody-producing capacity. It may be that there is a whole series of humoral agents concerned in the full development of the immune response (Janković, B. D., and Leskowitz, S. [1965]. *Proc. Soc. exp. Biol. Med.*, **118**, 1164; St. Pierre, R. L., and Ackerman, G. A. [1965]. *Science*, **147**, 1307).

Good: In one of Cooper's experiments (Cooper, M. D. *et al.* [1966]. *Science*, **151**, 471) chickens have been irradiated and bursectomized and then treated with fully-dispersed bursal lymphoid cells. These birds develop plasma cells and germinal follicles, but they cannot make antibody. It is possible that the bursal hormone enables this system of cells to receive information from either the macrophages or the thymic system; this concept could certainly be tested, as we have here a situation in which a hormone may be acting, and acting very quickly. St. Pierre and Ackerman (1965, *loc. cit.*) and Janković and Leskowitz (1965, *loc. cit.*) have presented some evidence in favour of the hormonal function of the bursa, as Sir Macfarlane has said, but bursal experiments are difficult to design well and bursal grafts are difficult—at least in the sites we have tried—to maintain. We have not been able to grow bursal tissue in Millipore filter chambers indefinitely. Dr. Peterson and others in our laboratory, however, have succeeded in fostering the development of visceral lymphomatosis by supplying animals with bursal grafts in diffusion chambers. I think there may be a hormonal effect here. It is even possible that in the chicken these two

systems are not absolutely separate. We could get more information about this by eliminating the thymus in young embryos; the result of such a manipulation might be a complete failure of development of the bursa and the bursal system, as well as of the thymic system of cells. The development of the whole of the lymphoid system may depend on a succession of steps which are revealed clearly in phylogeny and must also be gone through in ontogeny. If one step is not taken it is likely that all subsequent steps may be deficient.

Burnet: That is exactly what I am suggesting.

Nossal: There are three situations in mammals in which plasmacytopoiesis goes wild and antibody-producing potential is severely reduced. The first is in the neonatally thymectomized rat in which there is a very striking increase in plasma-cell proliferation with reduction of at least some of the parameters of antibody production. The second is exemplified by the rare type of thymic aplasia in human infants in which there is an associated alymphocytosis with normal γ-globulins and an extraordinary proliferation of plasma cells. We have seen one of these cases through the courtesy of Dr. C. Lee. This baby has normal levels of immunoglobulins yet his capacity to form antibodies is reduced although not completely absent. The third situation in which there is a tremendous increase in plasmacytopoiesis is in the post-irradiation state (Nossal, G. J. V. [1959]. *Aust. J. exp. Biol.*, **37**, 499). These findings are consistent with Good's idea that there are bursal and tonsillar systems, in birds and mammals respectively, which are responsible for the maturation and proliferation of plasma cells, and that there is also a thymic modulating factor which in some way gives to plasmacytopoiesis the drive towards antibody formation.

Mackay: Professor Good, what is your evidence that the tonsil in man serves the same purpose as the bursa in birds? If this were true then the widespread practice of tonsillectomy in children should be reviewed. The main evidence in favour of your "tonsil-

bursa-equivalence" hypothesis is that the tonsil is small or absent in agammaglobulinaemia. However, since the tonsils consist mainly of germinal centres, and a failure of plasmacytopoiesis would presumably be accompanied by failure of development of these germinal centres in the tonsil, this would in fact produce a small tonsil. Thus, small tonsils might be a result of the syndrome of agammaglobulinaemia rather than its cause. Professor Good, would you also elaborate your idea that auto-immune diseases are due to a failure of communication between the bursal and thymic systems?

Good: My ideas about the tonsil have been conditioned primarily by the histology of this organ in patients with the Bruton type of agammaglobulinaemia. We have now had considerable experience of tonsillar histology both in normal people and in patients with immunological deficiency diseases. In the Bruton type of agammaglobulinaemia we always find a failure of crypt and follicle formation, the morphology of the tonsil is lympho-epithelial and there are small accumulations of cells which look like thymic lymphocytes. This could be part of a general failure to develop follicles in all the lymphoid tissues, including the tonsils, but I think there is more to it than that.

There are other striking parallels between the bursa and the tonsil, for example the involutionary history of these organs. One of the easiest ways of recognizing last year's pheasants and thus picking this year's tender young birds is by the appearance of the bursa. This organ is about as big as the end of the thumb in this year's birds but is just a shrivelled up little bit of fibrous tissue in the year-old pheasants. One of the best ways of distinguishing between an 8-year-old child and a 25-year-old adult is by looking in the throat. In childhood the tonsils are large succulent lympho-epithelial organs like golf-balls, but they undergo a striking involution with sexual maturation. The involution of both the tonsils and the bursa precedes the involution of the thymus. There is also a structural similarity between tonsil and bursa:

in both organs the germinal follicles develop in intimate association with epithelium. And finally, phylogenetically, both tonsil and bursa seem to have appeared at about the same time in evolutionary history.

Our concept of the function of the tonsils is very important but it has been difficult to prove and experiments with the tonsil, like bursal experiments, are difficult to plan and execute. We have tried to irradiate monkeys and then remove their pharyngeal and palatine tonsils, but we have not been able to keep the animals alive with any regularity after these manipulations.

Dameshek: At what age do you take out the monkeys' tonsils?

Good: Timing may not be critical. In some of the experiments the animals have been immunologically mature at the time of our manipulations.

If auto-immune disease is caused by a failure of communication between the bursal and thymic systems, it is possible that a deficiency in the ability of the thymic system to organize and direct the immunological processes might contribute to such abnormalities as amyloidosis and Coombs' positivity.

Metcalf: There is no evidence whatsoever that the epithelial and reticular elements of the thymus direct the antigenic reactivity pattern of the body's lymphoid cells. If you transfer a thymus graft from animal (B) into a thymectomized animal (A), the restored animal (A) will continue to react to foreign antigens as an (A) animal. No one has ever shown any (B) reactivity in the (A) animals. (Globerson, A., and Feldman, M. [1964]. *Transplantation*, **2**, 212).

Good: Early (B) reactivity can be demonstrated (Miller, J. F. A. P. [1962]. *Ann. N.Y. Acad. Sci.*, **99**, 340; Dalmasso, A. P., *et al.* [1963]. *J. exp. Med.*, **118**, 1089). Identifiable donor cells are found in the spleens of neonatally thymectomized animals reconstituted with thymus from a foreign strain, but these cells do not persist for longer than about three weeks.

Metcalf: This is just due to the transitory migration of the (B) type of thymic cells into the animal. After three weeks, when these cells are lost, the animal reverts to its own original reactivity pattern. This suggests to me that the normal thymus does not dictate reactivity patterns and an abnormal thymus, therefore, would not be likely to dictate abnormal reactivity patterns.

Good: I was making this suggestion about a deficient thymus, not an abnormal one, since this is the current way of thinking about the thymus and its relationship to auto-immune disease. In rabbits subjected to thymectomy and appendicectomy in the newborn period we have found a fairly high incidence of a positive Coombs' test which implies that these animals have produced antibodies to their own red blood cells. Rabbits that have been irradiated, and thymectomized and appendicectomized immediately after irradiation when they weigh 1 kg., also developed auto-immune phenomena and amyloidosis. The animals became wasted and runted and quite regularly had Coombs' positive red cells. We do not yet know the meaning of the amyloidosis and Coombs' positivity. It is possible that these expressions of auto-immunity are induced by infection. Studies by Dr. Martin McKneally and Mr. David Sutherland in our department (unpublished) indicate that with antibiotic treatment the runting, the wasting and even the amyloidosis and the positive Coombs' test may be eliminated. McKneally suspects that the organism responsible for the infections in our colony is *Pasteurella multocida*, and it may be that infection with this organism induces a process that looks superficially like auto-immunity.

Metcalf: It was originally thought that runting after neonatal thymectomy was an auto-immune process due to thymic deficiency. This concept had to be discarded when it was found that thymectomized germ-free animals do not develop runting (McIntire, K. R., Sell, S., and Miller, J. F. A. P. [1964]. *Nature, Lond.*, **204**, 151; Wilson, R., Sjodin, K., and Bealmar, M. [1964]. *In* The Thymus: Wistar Institute Symposium Monograph No.

2, p. 89, ed. Defendi, V., and Metcalf, D. Philadelphia: Wistar Institute Press).

Good: The germ-free animal does not develop runting because it is not subjected to a constant input of antigenic challenges from the intestinal flora or from infecting organisms. In our colony of C3H mice neonatal thymectomy alone rarely gives rise to anaemia, amyloidosis or other manifestations of an auto-immune process. Yet in the same colony De Vries (De Vries, M. J., *et al.* [1964]. *Revue fr. Étud. clin. biol.*, **9**, 381) has shown, and Dr. Edmond Yunis has confirmed in our laboratory, that neonatal thymectomy of A strain mice yields 75 per cent with a positive Coombs' test, and there is a high incidence of amyloidosis; these animals have true haemolytic anaemia and they look sick as though they are being damaged by some intoxicating substance. Miller and his associates (McIntire, K. R., Sell, S., and Miller, J. F. A. P. [1964]. *Nature, Lond.*, **204**, 151) and Brother Wilson of Notre Dame (Wilson, R., Sjodin, K., and Bealmar, M. [1964]. *Proc. Soc. exp. Biol. Med.*, **117**, 237) have clearly shown that runting is eliminated by keeping neonatally thymectomized mice in a germ-free environment. Nevertheless, such experiments, important as they are, do not tell us if runting and wasting are due directly to infection, to intoxication from infecting organisms or even to inappropriate handling of antigenic stimulation by the neonatally thymectomized mice. Elimination of the micro-organisms does much more than just eliminate the possibility of infection and invasion of the tissues of these animals. It also eliminates the normal bowel infestations and the need for the mice to deal with a wide variety of antigenic or potentially toxic substances derived from microbial flora.

Metcalf: You are making the assumption that auto-immune reactivity against host cells is due to antigenic cross-reactivity between micro-organisms and host-cell antigens.

Good: Perhaps I am. It is easier to think of this as a cross-reactivity, or some kind of immune-absorption, immune-adherence

or even immunocomplex disease than as a direct assault against particular antigens of the host itself.

Dameshek: Does this apply to the NZB mice with auto-immune disease?

Good: I do not know. We should carefully consider the possibility that the pathogenesis of auto-immune disorder, the positive Coombs' test and so on, in the NZB mice may be based on infection.

Rowley: Professor Good, are you convinced that the differences in the results of bursectomy and thymectomy are not due entirely to quantitative differences in the immune response? Immunoelectrophoretic methods cannot detect antibody in animals which have been bursectomized and irradiated, but this may be because this technique uses quite large quantities of serum. Have you examined bursectomized and irradiated chickens after the induction of a graft-versus-host reaction at a stage when the host shows a stimulation of its reticulo-endothelial system, say at about ten days after the graft?

Good: Our current interest in, and research on, the bursal system and its relation to the thymic system stems from discussions I had with Warner in 1962 (Good, R. A., and Gabrielsen, A. E. [1964]. *In* The Thymus in Immunobiology, p. 413, *loc. cit.*). We cannot disregard the importance of quantitative factors in differences between the results of bursectomy and thymectomy, but the evidence is now substantial that there are morphologically identifiable lymphoid cells of two separate populations that can be related to two separate central lymphoid tissues and two separate functions. I do not know if these cells are qualitatively different from the original cells or not, but in the chicken they surely look different. On the other hand, in the lamprey, *Petromyzon marinus*, a primitive fish, both lymphopoietic and immunological functions appear together without the presence of an organ identifiable as thymus or bursa. Antibody production and cellular immunity have appeared, but there is no follicular or plasma-cell

system. It may be that the two separate functions appeared together and were the result of the biological processes involving one population of lymphoid cells. As evolution proceeded the two systems became separate and specialized, with the thymic system developing to completion at a more primitive stage than the bursal system, which first appeared among the primitive reptiles. By the time the evolutionary process has reached more recent creatures, such as the sharks and rays, the plasma-cell systems, as specialized cellular factories for γ-globulin synthesis, have already appeared. Basically, the two forms of immune response may be differentiations or specializations from the same potential. There do seem to be important quantitative and qualitative differences. For example, production of the bulk of antibody and the immunoglobulins depends on the plasma-cell system and later, phylogenetically, on the germinal-centre system as well. By contrast cellular immunity, whatever its chemical basis, remains a special province of the thymic system. I regard delayed allergy, part of homograft rejection and graft-versus-host reactions on the one hand, and immunoglobulin production and the synthesis of circulating antibodies on the other, as dissociable functions of two different systems of cells. These two systems appear in their final, fully differentiated form in mammals and birds where they have different end products, perhaps because their differentiation and specialization from a general capacity has proceeded so far. This does not, of course, mean that the cell systems are ultimately of separate origin and not related to one another.

Gowans: We have already discussed the possibility that a gut-associated hormone may be responsible in part for the achievement of immunological maturity by lymphoid tissue. The gut has a large area of associated lymphoid tissue and I wonder if during their recirculation through it lymphocytes may be influenced by another gut factor, namely endotoxin.

Good: Dent has shown (Dent, P. B., and Good, R. A. [1965].

Nature, Lond., **207**, 491), that the cells of the bursa—probably the most exposed of all the areas of lymphoid tissue—do not respond to stimulation by antigenic influences, including endotoxin, and, unlike spleen cells, seem incapable of plaque formation in the Jerne assay. The bursal cells seem aloof, in the same way as the thymic cells are aloof, from antigenic stimulation.

Gowans: Professor Good, will you elaborate on the conclusions you have drawn from your studies of bursectomy and thymectomy in fowls, and tell us how far you think it is legitimate to apply these conclusions to mammals? Are you suggesting that in mammals there is a follicular system of large cells which mediates antibody formation, and another developmentally separate system which mediates transplantation reactions?

Good: The regular morphological association of cells containing immunoglobulins and antibodies and an organ containing what we believe to be precursor cells that themselves have a functional deficiency of the parameter under investigation, indicates to me that this is a specialized system whose specialization is due to the differentiation of its cells. Similarly in the thymus, the direction of differentiation leads to an end-stage cell that functions in cellular immunity, but feebly if at all in humoral antibody synthesis. Delayed sensitivity, homograft rejection and the graft-versus-host reaction are anatomically intimately linked with the thymus, both in clinical and experimental situations; immunoglobulin production and the cells related to this by more direct means are intimately linked to the bursa. I do not believe that our observations absolutely exclude the development of the thymic system of cells into antibody-producing cells, but our findings do seem to render this an infrequent event under normal physiological circumstances.

Dameshek: That is in the chicken. Does the same apply to all animals, including mammals? In man, for instance, does the thymus perhaps combine all these various functions?

Good: We do not yet know for sure what happens in mammals.

Our best evidence for the existence of two systems in man comes from our observations of the several experiments of nature which I have described (this volume, p. 199, 468). Firstly, there are patients with no small lymphocytes in the circulating blood, but with normal γ-globulins, a well-developed plasma-cell system and some ability to form antibody. Secondly, there is the syndrome in which there is a normal thymic system and a normal blood lymphocyte level and general capacity to develop the delayed allergic responses, graft-versus-host reactions and homograft rejection, but in which the patients cannot produce antibody. Finally, there are conditions in which both systems are inactive. When we have found a way to reconstitute these deficiencies in man we will probably have the answer to many of the major problems of immunobiology.

PARALLEL OBSERVATIONS ON THE ROLE OF THE THYMUS IN LEUKAEMOGENESIS, IMMUNOCOMPETENCE AND LYMPHOPOIESIS

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The thymus contains an agent or agents that can restore to thymectomized hosts their sensitivity to leukaemogenic agents (the thymic leukaemogenic factor). The objectives of our studies have been to determine the nature of this material and its relationship to the factors responsible for immunological competence and lymphopoiesis. The thymic leukaemogenic factor is probably non-cellular, since thymic grafts can induce lymphoma in the cells of their host. The factors inducing immunocompetence and stimulating lymphopoiesis are probably also non-cellular, as shown by the effects of thymic grafts in Millipore filter chambers and the stimulating effect of cell-free extracts of the thymus on lymphopoiesis (see Kunii, Furth and Berwick, 1966).

Recent immunological studies indicate that leukaemogenic viruses are widespread among mice but that the virus discovered by Gross (1951) is unique among them because it causes "spontaneous" leukaemia in the so-called high-leukaemia strains of mice (Old, Boyse and Stockert, 1965) and because it has a specific relation to the thymus.

Almost all the reported work on the thymic leukaemogenic factor has been done in mice. We have studied this factor in the highly inbred W/Fu strain of rat in which leukaemia is very rare.

The wild type of Gross virus occurs naturally in several strains

of mice, but not in rats. We adapted it to W/Fu rats about five years ago and found that the lymphomas it induces in these rats are similar in many ways to the lymphomas it induces in mice. There are, however, basic differences in the reaction of AK mice and W/Fu rats to this virus: the normal rat is free from it—in the AK mouse it is congenital; the mouse, as an embryo, is exposed to the virus and so the adult mouse does not recognize the virus as alien—the rat does; the rat, therefore, can be readily immunized to the leukaemias induced by virus—the AK mouse cannot; all leukaemias arising spontaneously in AK mice could be grafted on to AK hosts—many of the virus-induced lymphomas in W/Fu rats are incompatible with the strain of origin.

The evidence that the high-leukaemia strains of mice carry the Gross virus is (a) that there is vertical transmission of the virus in thymectomized hosts (Furth, 1946; Gross, 1961); (b) that characteristic viral particles are present in germ-free mice of the high-leukaemia strains (DeHarven, 1964; Pollard and Matsuzawa, 1964); and (c) that Gross antigen (G-antigen) is present in these mice (Old, Boyse and Stockert, 1965). Old and co-workers found that normal W/Fu rats are free from G-antigen but that they acquire it when they are infected with Gross virus. Thymic lymphomas and other types of leukaemia occur spontaneously in W/Fu rats but there is no evidence that they carry a leukaemogenic virus, and Old did not find any G-antigen in the rats with lymphoid tumours. We have tried for many years, without success, to develop a high-leukaemia line of the W/Fu strain of rat in the same way as has been done with AK mice. We attribute our failure to a lack of leukaemogenic virus in these rats.

There are several technical advantages in studying the thymic factors of viral leukaemia in W/Fu rats: the end-points and base-lines are sharp; spontaneous leukaemia is practically non-existent during the first year of life but almost all rats infected neonatally with Gross virus die with lymphoma about 3 to 4 months later; the induction rate of lymphoma is nearly zero in well-thymectom-

ized rats; large quantities of material can be obtained for culture and viral studies—notably pure viral pellets from plasma—and this can be assayed for the presence of virus in mice without the strict control of eliminating the cells in the inoculum by filtration and other means, a process which is usually accompanied by a reduction in the viral activity of the preparation; fertility of W/Fu rats is high and thymectomy is more easily performed in them than in the AK mice.

THYMIC LEUKAEMOGENIC FACTOR

We were unsuccessful in our attempts to study the thymic leukaemogenic factor by utilizing thymic grafts in Millipore filters and so we returned to the now classical technique which utilizes genetic markers. Strain chromosome markers are not known in rats. The W/Fu rat contains a readily identifiable Y chromosome (Zieverink and Moloney, 1965) which may possibly serve as a marker if the sexes of donor and recipient are different. Female W/Fu rats accept thymic grafts from males. To distinguish between the lymphoid cells of host and graft the virus was injected into $(W/Fu \times F/Fu)F_1$ hybrids. Both these strains are highly inbred and the two parental strains are histologically incompatible. The thymus grafts were derived from the parental strains. We learned through trial and error (*vide infra*) that in the study of thymic leukaemogenic factor it is essential to thymectomize the rat before infecting it with virus. Such a procedure is obviously impossible in mice carrying the leukaemia virus. In studies of the thymic leukaemogenic factor, thymectomy can be and is more conveniently performed when the animals are several days old. In contrast, in studies of the immunocompetence and lymphopoiesis stimulating factors, thymectomy is best performed during the first day of life.

In our first experiment (Kunii and Furth, 1964), virus was injected during the first three days of life and thymectomy was

performed about three weeks later. Several well-thymectomized rats in this series developed leukaemia. We assumed that this was because some thymic cells underwent lymphomatous transformation during the first three weeks of life and colonized in distant organs. Therefore, in all subsequent experiments, thymectomy preceded the injection of virus. The virus was usually injected on the day of thymectomy and few or none of the rats treated in this way developed leukaemia (Kunii, Furth and Berwick, 1966). The incidence of infection at operation sites, common in earlier experiments, was greatly reduced by the development of a thymectomy snare (Kunii, Furth and Berwick, 1966).

RELATION OF THE NUMBER OF THYMIC GRAFTS TO RESTORATION OF SENSITIVITY TO VIRUS

In attempts to enhance the sensitivity of leukaemia to the virus, we made use of Metcalf's (1963) observation of the autonomous behaviour of thymic grafts. As Fig. 1 indicates, restoration was more successful with multiple than with single thymic grafts.

In this series several thymuses were grafted at one site. They did not behave alike, some involuted and some became lymphomatous in focal areas (Fig. 2). The largest tumour arising in a graft weighed 60 gm. (Kunii, Furth and Berwick, 1966).

In all our later experiments thymic tissue was grafted at several sites and the behaviour of these grafts also varied greatly. At one extreme, most thymic grafts were lymphomatous and, when thymectomy was incomplete, the residual thymus was also lymphomatous (Fig. 3). One is tempted to conclude from the study of the rat shown in Fig. 3 that leukaemogenesis is multicentric, and the most aggressive neoplastic cell in this rat arose in the graft on the right groin and caused massive metastases in the regional (iliac) lymph nodes. Multicentric transformation is as conceivable as unicentric transformation and both events may occur in the same host. This situation has not been fully resolved. Law

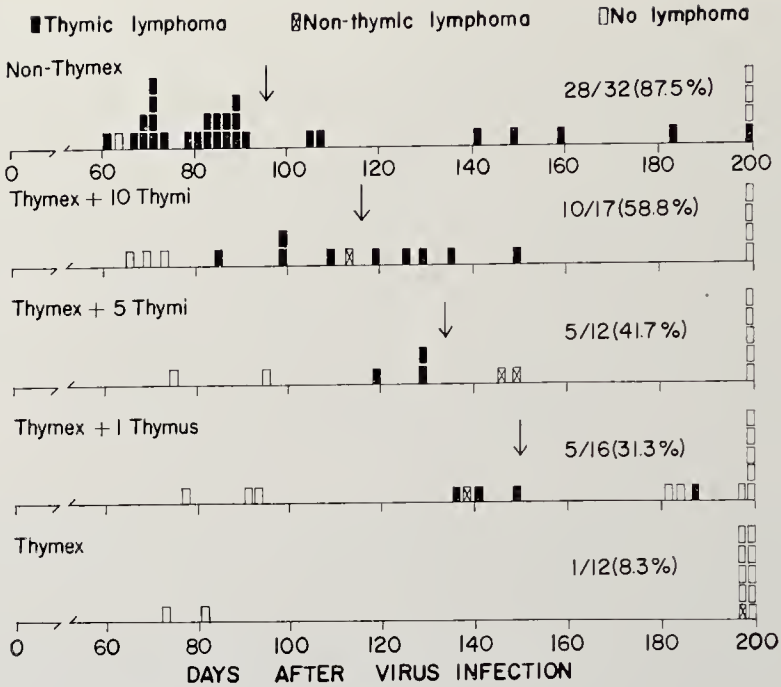

FIG. 1. Histogram of leukaemias in thymectomized rats with 1 to 10 thymus grafts, and in controls. Thymex = thymectomized rats. Thymi refers to the number of thymuses that were grafted.

and Potter (1956) have shown that some of the lymphomas arising in a thymus graft are of the host-cell type. We were much impressed by the large number of leukaemic cells present in the circulating blood when the disease was generalized and most grafts were lymphomatous. The normal thymus is not a favoured site for metastases which is a helpful distinction in deciding whether an experimental lymphoma is induced by virus or by grafted cells.

In another extreme case of a well-thymectomized rat only one of the five grafted thymuses developed lymphoma. The grafts at

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FIG. 2. Multiple thymic grafts in a thymectomized virus-infected rat. (a) Survey (× 10); (b) an atrophic and a normal looking thymus (× 100); (c) a leukaemic thymus (× 100).

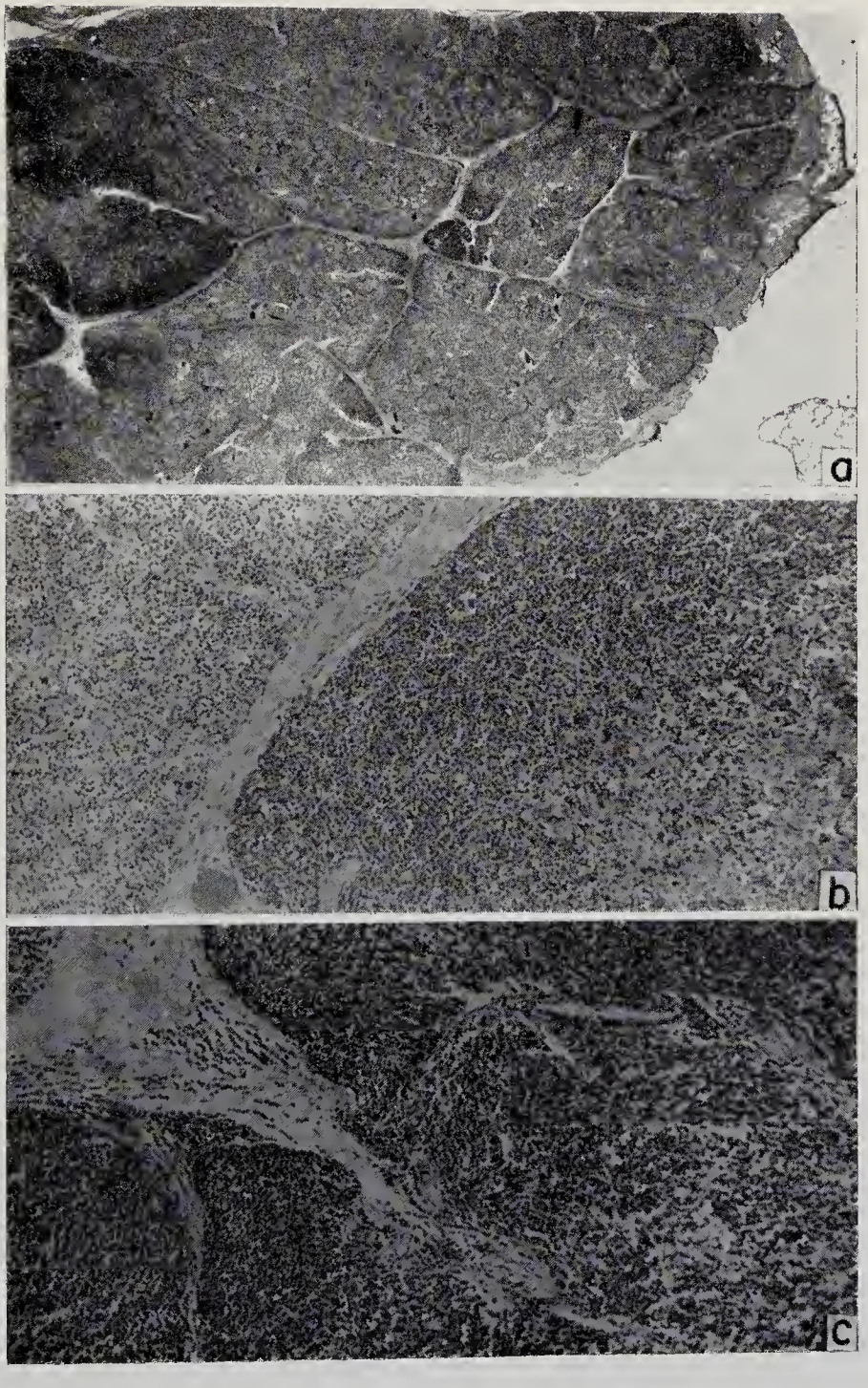
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FIG. 3. Thymectomized virus-infected rat with multiple thymus grafts. The largest lymphoma, in the right groin, replaced a graft and caused extensive metastases in the lumbar region.

other sites were enlarged but microscopically hypofunctional as were the lymph nodes and other blood-forming organs. In this case neoplastic transformation during the period of observation occurred at only one site and the neoplasia remained localized there.

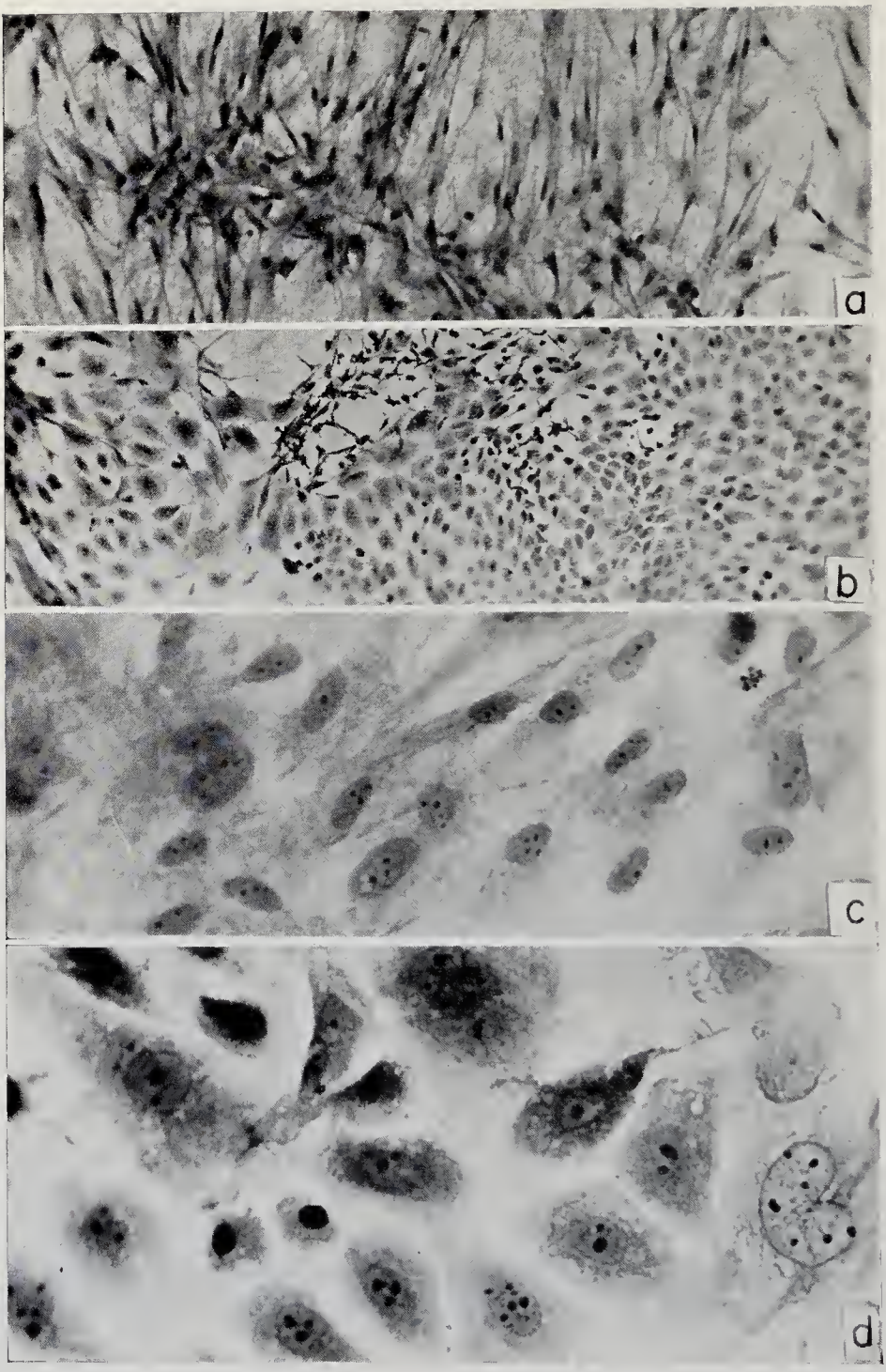
Transplantation studies of 14 lymphomas arising in thymectomized F_1 rats grafted with parental thymuses suggest that some of the lymphomas originated in the host and some in the donor cells. Some of the tumours were histologically incompatible with both host and donor cells and these grew on newborn rats and either killed the hosts soon after grafting or regressed (Kunii, Furth and Berwick, 1966).

Old and Boyse (1965) assayed G-antigen in the blood, spleen and lymph nodes and in lymphomas arising in thymic grafts in this series. They found most of the antigen in the tumours and spleens of the lymphomatous rats, and some in the plasma. This antigen was also found in small concentrations in infected rats which had failed to develop frank lymphomas, but not in normal rats nor in animals with the transplantable spontaneous leukaemias that Dr. Boyse has described in older rats of the W/Fu strain.

Old, Boyse and Stockert (1965) found that there is relatively little G-antigen in the thymuses of normal rats but more G-antigen is present in cells with a reticulo-endothelial function, and in the lymphomatous thymus. These observations do not support the idea that the thymus is a favourite site for replication of the Gross virus, although it is certain that virus accumulates in the thymus in large concentrations when this organ becomes lymphomatous (Okano, Kunii and Furth, 1963).

RELATION OF THE THYMIC LEUKAEMOGENIC FACTOR TO EPITHELIAL-RETICULAR CELLS

The reported failure to restore leukaemia sensitivity to Gross virus by thymic extracts (Miller, 1961) and thymic lymphocytes (Miller, 1964) led us to attempt to restore this function with thymic reticulum cells, the presumed source of the presumed thymic factors. As a matching control, thymic lymphocytes were injected into thymectomized hosts. Although these experiments are still in progress it seems certain that epithelial-reticular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cells possess the leukaemogenic factor. Among the several conceivable procedures for obtaining lymphocyte-free epithelial-reticular cells, the tissue culture technique appeared the most promising.

Our cultures of thymic epithelial-reticular cells originated in normal one to six-day-old W/Fu rats of both sexes. The thymic "mince" was placed on chicken plasma drops. Beef embryo extract was used to clot the drops. The cultures were grown in Puck's medium supplemented with 15 per cent foetal bovine serum which was changed twice weekly. The incubator contained 5 per cent carbon dioxide in air. After about twenty days, when viable lymphocytes were no longer observed, the cultures were gently scraped, pooled and centrifuged at 800 revolutions per minute for five minutes. The plasma pellet was resuspended in a small volume of its own supernatant fluid and injected into the thigh muscle of neonatally thymectomized rats.

At first the cultures were composed almost exclusively of floating small lymphocytes. With the twice-weekly change of medium, the lymphocytes became less and less numerous and larger cells formed a continuous layer (Fig. 4). In stained preparations "viable" lymphocytes were rare in cultures older than three weeks. The cells forming the culture varied in different areas and in different cultures. Some cells were fibroblast-like, others more plump and stellate, and often anastomosed in a reticulum; still others—the least common—had an epithelial appearance. Although there appeared to be transitional forms between the different cell types, it is unlikely that these cultures originated from a single cell type.

Grafts of cultured normal thymic-reticulum cells induced a large lymphoma at the site of the graft in two rats in which a

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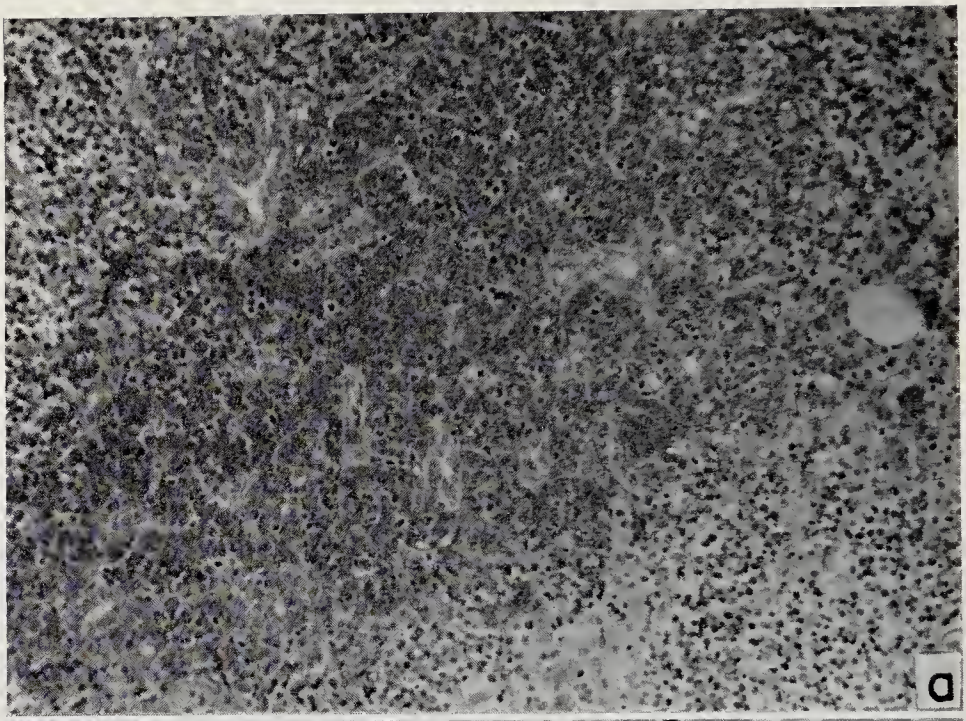
Fig. 4. Appearance of epithelial-reticular cell cultures of the thymus at different magnifications: (a) × 140. (b) × 140. (c) × 400. (d) × 400.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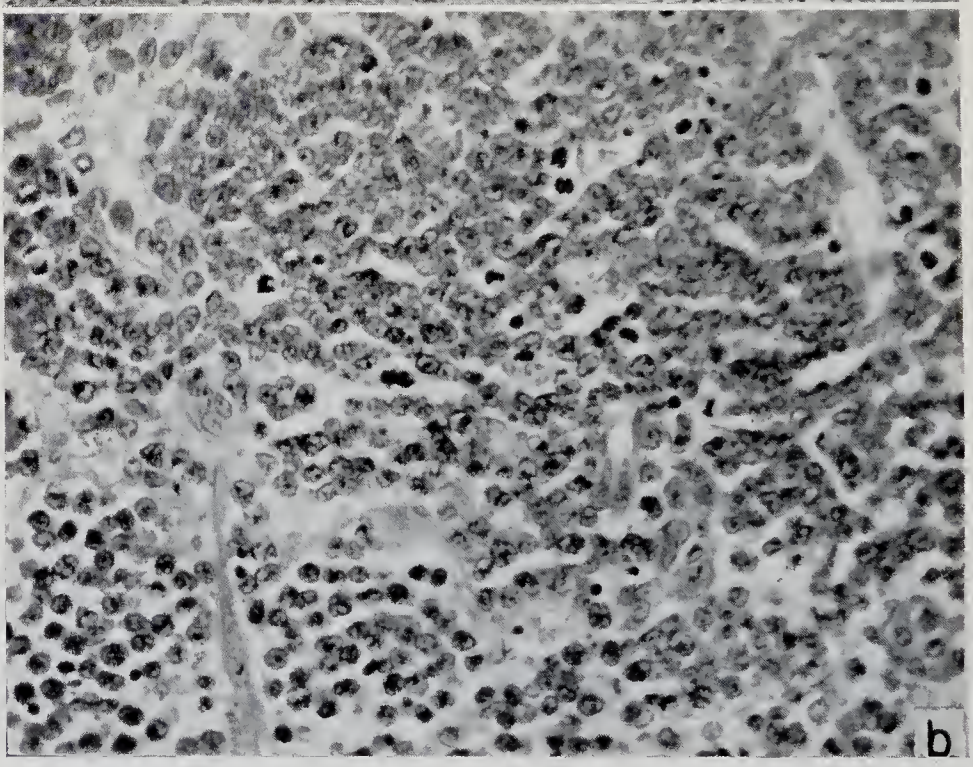
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thorough gross and microscopic study confirmed the completeness of thymectomy. Large lymphomas were demonstrated macroscopically and microscopically at the graft site in two other rats in which autopsy disclosed thymus remnants.

The microscopic appearance of a tumour arising in epithelial-reticular cells at the site of the graft in a well-thymectomized rat is shown in Fig. 5. The dense core of tumour consists of neoplastic cells in contact with each other and forming gland-like structures strongly suggestive of transformed epithelial-reticular cells. Surrounding this centre of epithelial-like cells are large and medium lymphocytes freely infiltrating the muscle. Mitotic figures are abundant in both types of cell. Several rats in this series had generalized lymphoblastic leukaemia, but the lymph nodes were largest and the leukaemic infiltrations most massive at the site of the graft.

The cultures may have contained a few lymphoblasts but it has been shown that even large numbers of thymic lymphocytes fail to restore the sensitivity of rodents to leukaemogenic agents (Miller, 1964). Further, the microscopic study of the grafts showed an intimate relation of the lymphomas to the epithelial-reticular cells. Grafts which failed to develop lymphomas within the period of observation showed a remarkable reconstitution of the grafted epithelial-reticular cells, often with the formation of an atrophic thymus-like nodule containing abundant epithelial-reticular cells.

Cytogenetic assays of the cell type, now in progress, are expected to yield further evidence of the grafted epithelial-reticular-cell origin of these lymphomas. We hope to learn more about the relationship of the epithelial-reticular to the lymphoma

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FIG. 5. Tumour produced at the site of a graft in the thigh, with epithelial-reticular cells. Note epithelial-like cells in the centre with (leukaemic) lymphocytes around them. (a) × 150. (b) × 400.

cells by studies with radioactively labelled epithelial-reticular cells. The epithelial-reticular-cell tumours induced by polyoma virus may prove to be functional and useful in such studies. It is remarkable that Gross virus does not induce epithelial-reticular-cell tumours of the thymus and that the polyoma virus does not induce thymic lymphomas.

Previous studies suggest that the epithelial-reticular cells of the thymus are the source of lymphopoiesis stimulating factor and thymic leukaemogenic factor and electron micrographs of tumours arising in thymic grafts support this concept. Budding forms of virus are more common on the villous processes of cells —probably epithelial-reticular cells—than on the plasma membrane of lymphoid cells and within intracytoplasmic vacuoles (Kunii, Furth and Berwick, 1966). Most virus is seen intercellularly, as was noted by Okano, Kunii and Furth (1963) in lymphomas arising in thymuses in their normal location. Extracellular accumulation of virus seems greatest in areas of necrosis.

Are the epithelial-reticular cells the ancestral cells of the normal thymic lymphocytes? It appears from our studies that in rats infected with virus the reticulum cells themselves become transformed and differentiate into, or otherwise induce the formation of, neoplastic lymphoid cells capable of unrestrained proliferation. It should be recalled in this connexion that attempts to grow normal lymphoblasts have thus far been unsuccessful, while leukaemic lymphoblasts can be maintained *in vitro* for several years (Ioachim, Berwick and Furth, 1966).

During embryonic development the primordial lymphoblasts are said to arise from epithelial reticulum (Auerbach, 1961). Sainte-Marie and Leblond (1964) also traced thymic lymphocytes in adult rats to the "reticular" cells. It is not known if the postnatal thymic epithelial-reticular cells form lymphoblasts but it is possible that this transformation may occur under the stimulus of a virus. Another possibility is that the epithelial-reticular cells merely attract and stimulate lymphopoiesis. In our future

experiments with cultured epithelial-reticular cells labelled with tritiated thymidine we will investigate the behaviour of neoplastic lymphoblasts at graft-sites near to the epithelial-reticular cells. If these malignant cells take up the label it will suggest that they are derived from epithelial-reticular cells, but if they do not this will suggest that the epithelial-reticular cells simply stimulate lymphopoiesis.

The induction of lymphomas by epithelial-reticular-cell grafts recalls the isolation of two transplantable sarcoma-like tumours at the site of thymus grafts in thymectomized AK mice (Furth, 1941). These experiments were done before the leukaemia virus was known and the purpose of the work was to find out if an exteriorized thymus would develop a thymic tumour. These sarcoma-like tumours were carried in numerous passages and were thought to be thymomas. A number of organs grafted at abnormal sites can give rise to tumours and some of these are functional. Since it is possible that some thymic epithelial-reticular-cell tumours are also functional, attempts to develop them may be useful.

THE RESTORATION OF LYMPHOCYTE LEVELS AND IMMUNOCOMPETENCE IN THYMECTOMIZED RATS

Thymectomy during the neonatal period causes a lasting decrease of lymphocytes in the blood. In preliminary experiments, the lymphocyte levels in the blood of thymectomized uninfected rats which had been implanted with one to ten thymus grafts were studied for 160 days. The results of these experiments (Fig. 6a) indicate that the degree of restoration of the blood lymphocyte level is directly related to the number of thymuses grafted (Kunii, Furth and Berwick, 1966). Thymectomy was usually performed on the third postnatal day. In an earlier experiment in which thymectomy was performed on the first day of life the lymphopenia was more profound (Fig. 6b).

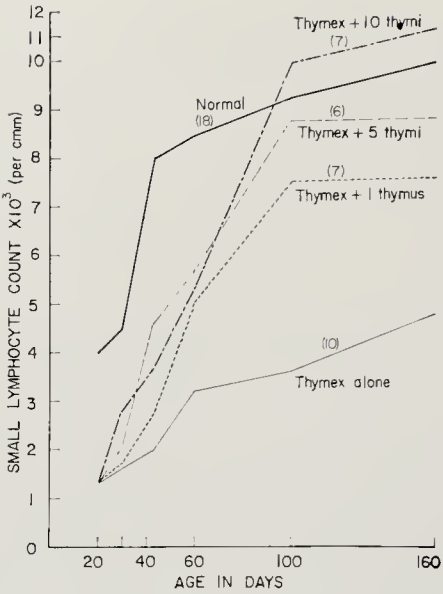
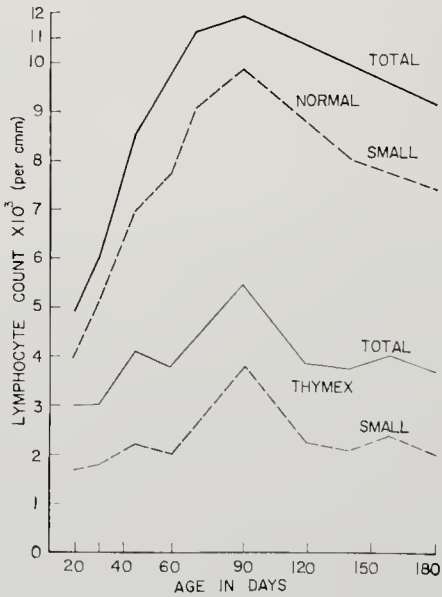


FIG. 6(*a*)



(FIG. 6*b*)

FIG. 6. Blood lymphocyte levels of thymectomized rats. (*a*) at about 3 days; (*b*) at about 1 day. Thymex = thymectomized rats. Thymi refers to the number of thymuses that were grafted.

Agglutinin titres to a single injection of sheep erythrocytes were determined in virus-infected and non-infected rats of the same series. The haemagglutinin data (Table I) do not quite parallel the data on the effect of neonatal thymectomy on the lymphocytes. The corrective effect of one thymus graft on the post-thymectomy deficiency of immunocompetence factor was about the same as the effect of five to ten thymus grafts on the post-thymectomy lymphopenia. Lymphopenia was much more profound in virus-infected than in normal rats and, in this small series, it was not well corrected by thymus grafts.

TABLE I

AGGLUTININ TITRES TO SINGLE INJECTION OF SHEEP ERYTHROCYTES

Rats	*Non-infected*	*Virus infected*
Normal	3062 (7)‡	384 (2)
Tx†	439 (7)	64 (2)
Tx+1 thymus	2460 (6)	256 (1)
Tx+5 thymuses	2554 (4)	601 (3)
Tx+10 thymuses	2573 (6)	384 (2)
Age when tested:	160–170 days	200 days

† Thymectomized (neonatally) None of these rats had leukaemia .
‡ Figures in parenthesis give the number of rats in the group .

These preliminary studies suggest similarities in the behaviour of the three thymus factors under investigation, but their parallel behaviour merely indicates that neonatal thymectomy makes the host deficient in all three factors and that thymus grafts restore the deficiencies.

Our present experiments, in which individual rats of the various groups are tested during the preleukaemic period for the presence of these three factors, were designed to demonstrate some discrepancies which we had anticipated might occur. We compared the restorative effect of the following tissues on thymic deficiencies: newborn with adult thymus grafts, epithelial-reticular cells of the thymus with thymic lymphocytes; and thymus with spleen grafts. The preliminary results of these

experiments, in which haemagglutination tests and lymphocyte counts were made during the preleukaemic period, disclosed extreme variations within the group. For example, some rats which ultimately died of viral lymphoma had a very low, and others a high, haemagglutinin titre, whether they had received a thymus graft or not. This was also true for the rats which failed to develop lymphoma. In general, virus alone depressed the blood lymphocyte levels and this effect was magnified by thymectomy and minimized by thymic grafts. But our results showed such marked variations and extreme divergences that we have had to conclude that either the factors themselves, or the mechanisms by which they operate, are markedly different.

DISCUSSION

Analysis of these observations indicates that the three functions of the thymus are separate though intertwined.

Thymectomy of young adult mice prevents the spontaneous development of the leukaemia caused by the wild type of Gross virus which these mice harbour. The Gross virus becomes a mere passenger in these mice and causes only slight lymphopenia and minimal, if any, diminution of immunological competence. This divergence in the results is best explained by supposing that the production of thymic leukaemogenic factor is not linked to the production of the immunocompetence or lymphopoiesis stimulating factors. In other words, even the mature thymus which lacks immunocompetence factor possesses thymic leukaemogenic factor.

During the early neonatal period differentiation of immunocompetent lymphocytes in the thymus appears to stop although the ability of the thymus to produce lymphocytes and to secrete leukaemogenic stimulating factor continues (Metcalf, 1956). In rats and mice the ability to produce, by differentiation, immunocompetent lymphocytes is almost monopolized by the neonatal

thymus but this is not the case for lymphopoietic capacity which goes on in all lymphoid tissue. The thymus is not the sole site of formation of malignant lymphocytes. However, the ease of induction of lymphomas in rats and mice by Gross virus *is* linked to thymic activity. Why is the thymus usually the primary site of leukaemia? There are several possible explanations for this: the concentration of lymphopoiesis stimulating factor is greater in the thymus than anywhere else in the body and the thymus may have a specific affinity for the virus.

The thymic leukaemogenic factor is best understood by analogy with the promotional role of hormones in carcinogenesis, for example, in mammary carcinoma. In both mammary cancer and leukaemogenesis in the thymus a resident, apparently harmless, virus can be transmitted vertically and the hormonal stimulation of its target organ is the determining factor for the induction of neoplasia.

Hormones which stimulate an organ usually promote carcinogenesis in that organ. Our knowledge of the secretions of the thymus and its homoeostasis is still fragmentary and inferential. Adrenalectomy stimulates the lymphopoietic activity of the thymus (Dougherty, 1959) and promotes leukaemogenesis (Law, 1947). Corticosteroids and agents causing thymic involution depress leukaemogenesis.

Analysis of antigenic and genetic modifications are beyond the scope of our present experiments. However, it has been established that some viral lymphomas in the rat are antigenically modified and become histologically incompatible with their hosts (Kunii, Furth and Berwick, 1966) and that the infected rats carry the G-antigen (Old and Boyse, 1965). The chemical properties of this antigen and its relation to the Gross virus remain to be determined.

Leukaemia induced by the same virus in the rat and mouse varies greatly but each type can be perpetuated by cell passages. Of great interest are the two extreme types: in one of these the

white cell count is several hundred thousand and tumour formation is absent and in the other grafted tumours are huge and the blood is not leukaemic. Now that auto-antibodies can be readily assayed the old idea (Furth and Baldini, 1953) that the aleukaemic type of lymphoma is due to an antigenic modification resulting in high levels of auto-antibodies can be tested. Although the non-cellular factors under discussion are best considered as fluid hormone-like substances secreted by the thymic reticulum, this concept is inferential and requires solid evidence to confirm it. For instance, the possibility that genetic information from the polynucleotides of the virus is incorporated into the host lymphocytes may be remote but has to be excluded. It should also be recalled that even inert plastic of definite physical structure can induce neoplasia (Alexander and Horning, 1959).

CONCLUSION

These are our present concepts on the interrelationship of the three thymic factors. They have been formulated by filling in the gaps in our knowledge with speculations.

(1) Immunocompetence is related to a unique type of differentiation which normally ceases during late gestation or in the early neonatal period. It produces a specific thymic cell type, the new self-perpetuating lymphocyte—the immunocyte.

(2) The lymphopoiesis stimulating factor, in contrast, persists throughout life, but diminishes with age as other lymphopoietic organs become capable of maintaining lymphocyte homoeostasis in the absence of the thymus.

Both these factors are presumed to be cell-free and to function as organizers (inducers); that is, they can pass through cell-tight filters. The presumed source of the lymphopoiesis stimulating factor is the epithelial-reticular cell.

(3) The thymic leukaemogenic factor is linked to both the immunocompetence and the lymphopoiesis stimulating factors.

In the process of differentiation, as well as during replication, cells are vulnerable to carcinogens. The thymic leukaemogenic factor operates at times when the thymus is highly active in lymphopoiesis and this factor's action is visualized as hormonal. Stimulation of lymphopoiesis by any agent promotes leukaemogenesis.

The existence of high-leukaemia strains of mice is due to the congenital presence of a leukaemogen in these mice—the Gross virus "vertical transmission". The lymphopoiesis stimulating and the immunocompetence factors operate in mice of both high-leukaemia and low-leukaemia strains. When the lymphopoiesis stimulating factor is hyperactive, leukaemogens can induce lymphoma in low-leukaemia strains of mice at a rate comparable to the rate at which these tumours occur spontaneously in the high-leukaemia strains. A leukaemogenic virus is an excellent agent to accomplish this induction. Without the host factors that stimulate lymphopoiesis, the virus, as other carcinogens, appears to be harmless. It can become a lifelong passenger in the host if it is present during the embryonic period or a transient passenger if infection occurs at a time when the host is immunologically competent.

SUMMARY

Studies of experimental leukaemia induced by the Gross virus in W/Fu rats confirm and extend the basic knowledge obtained from studies of mouse leukaemia.

The behaviour of the three presumably hormone-like factors of the thymus, which are related to leukaemogenesis, lymphocyte production and immunocompetence respectively, is parallel in some ways but markedly divergent in others. This suggests that there are basic differences between the three factors. Thymectomy in the neonatal period causes a roughly parallel deficiency in all three functions and the severity of the deficiencies is dependent mainly on the age of the rat at thymectomy. All

functions can be corrected by thymus grafts and the degree of repair is roughly proportional to the quantity of thymic tissue grafted.

Grafts of cultured thymic reticulum cells can restore the leukaemogenic deficiency of thymectomized hosts. Our observations suggest that epithelial-reticular cells can either be transformed under the stimulus of the virus and differentiate into lymphocytes, or that they can induce the formation of attracted lymphoid cells.

Rats infected with Gross virus acquire the Gross antigen. Transplantation studies indicate that some lymphomas originating in rats infected with virus belong cytogenetically to the host and others to the donor, but that most of these tumours are histologically incompatible with both donor and host.

In the high-leukaemia strain of AK mice, but not in W/Fu rats and some other mouse strains, the Gross virus is congenital and consequently the hosts are tolerant to it. Parallel studies of the pathogenic events leading to the development of virus-induced leukaemia in two basically different types of hosts—AK mice and W/Fu rats may lead to a better understanding of the possible role of viruses in the induction and control of leukaemia in man.

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INTERACTION OF OCCULT LEUKAEMOGENIC VIRUSES WITH IONIZING RADIATION AND OTHER EXTERNAL LEUKAEMOGENIC AGENTS IN THE INDUCTION OF THYMIC LYMPHOSARCOMA IN THE MOUSE

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Ionizing radiation and a variety of chemical agents have long been known to induce lymphosarcoma and lymphatic leukaemia in the mouse (Kaplan, 1957; Miller, 1961). These neoplasms which have a remarkable propensity to develop in a single site of origin—the thymus—have been among the most interesting and fruitful test-systems for the study of carcinogenesis.

About ten years ago a paradoxical phenomenon was observed: when normal strain C57BL thymus glands were implanted subcutaneously into thymectomized, irradiated host mice, a significant proportion of the ensuing lymphoid neoplasms developed from the cells of the graft, even though these cells had never been exposed to X-rays (Kaplan, Hirsch and Brown, 1956; Law and Potter, 1956; Barnes *et al.*, 1959). Meanwhile, the work of Gross (1951, 1954) had called attention to the existence of leukaemogenic viruses in the "spontaneous" lymphomas of certain high-leukaemia strains of mice. A search was therefore made for a similar agent in our system. By 1957, preliminary evidence indicated that cell-free extracts from radiation-induced lymphomas of strain C57BL mice contained an agent capable of

inducing identical tumours after inoculation into non-irradiated, newborn mice of the same strain. Cell-free extracts of the agent-induced lymphomas were active on serial passage and could be shown to induce tumours *de novo* rather than by transfer of viable neoplastic cells (Lieberman and Kaplan, 1959). Similar results were independently obtained by Gross (1958) in the C3H strain, and confirmation of our observations has been reported by Latarjet and Duplan (1962), Ilbery and Winn (1964) and Libánský and Láznička (1964).

Although the preliminary evidence suggested that the agent obtained from radiogenic lymphomas was probably a virus, several years of arduous effort have had to be devoted to the task of establishing this with greater certainty. In this report I should like to summarize what we have learned to date about the radiation leukaemia virus (RLV), about its interaction with radiation and other external leukaemogens, and about the pathogenesis of the tumours it induces.

EVIDENCE OF VIRAL NATURE OF THE LEUKAEMOGENIC AGENT IN EXTRACTS OF RADIOGENIC LYMPHOMAS

The difficulties of working with this agent, initially, were enormous. Activity was detectable in only a small fraction of the radiation-induced tumour extracts tested, and even the active extracts, when injected into neonatal, C57BL recipients, yielded a relatively low incidence of tumours (about 15 to 20 per cent) and this after a discouragingly long latent period (mean: 388 days). Unfortunately, most of the mouse leukaemia viruses are not efficiently propagated *in vitro*, nor do they induce cytopathic effects which can be used as the basis for an *in vitro* assay. It was therefore necessary to build up the potency of the RLV *in vivo*, by serial passage from virus-induced tumours into newborn C57BL mice. Although this procedure was ultimately successful, it required many more passages than in similar efforts with

the Gross (1957) and Moloney (1960) viruses. The original RLV material is now in its twenty-fourth consecutive passage and complete data on the first twenty passages are available. In the first ten passages lymphoma incidence induced by RLV was about 40 per cent, with an average latent period of about 190 to 200 days (Kaplan, 1964). Since that time there has been a gradual increase in incidence and reduction in latent period. In the nineteenth serial passage, for example, the seven cell-free extracts tested yielded lymphomas in 86 of 92 C57BL mice (93 per cent), with an average latent period of 152 days.

During the course of the serial passages some of the extracts were also inoculated into newborn mice of other strains and of various hybrid combinations to obtain information on the host range of the virus. Additional host-range studies are currently in progress. RLV differs from the Gross virus in the following respects: it is most active in strain C57BL, in which Gross virus is inert; it exhibits only slight activity in strain C3H mice, which are highly susceptible to Gross virus; it is consistently active in BALB strains of mice, though less so than in C57BL mice; and, although the serially passaged RLV preparations mimic the accelerating activity of the Gross virus after neonatal inoculation into AK mice (Rudali, Duplan and Latarjet, 1957; Kaplan, 1964), this phenomenon is not exhibited by cell-free extracts prepared directly from radiogenic lymphomas of strain C57BL mice. Although immunological studies have not yet been carried out, RLV is distinguishable from Moloney virus (Moloney, 1960) by its much more restricted host range and the much longer latent period required for tumour induction.

Modest progress has been made in our attempts to purify RLV. Differential centrifugation of homogenates of lymphoma tissue in phosphate-buffered saline yields activity in the cytoplasmic material which pellets at 10,000 to 30,000 **g**. The activity withstands extraction by fluorocarbon but is destroyed by ether,

ethoxy-ethanol, and other lipid solvents, by protamine, and occasionally by trypsin. Activity is maintained after storage at temperatures of liquid nitrogen. Cytoplasmic pellets obtained from serial-passage-induced lymphomas were active after five hundred to a thousandfold dilution. An experiment to determine the dilution end-point titre of our present preparations is in progress.

Preliminary attempts have been made to purify RLV from cytoplasmic pellets further by density gradient centrifugation in potassium citrate or tartrate. In most of these experiments a "milky" band has been observed at a density of about 1·15 with a more granular band at a slightly heavier density. The latter band has in some instances been dissociated into two bands. Maximal leukaemogenic activity has usually been associated with the milky band but appreciable activity has in some instances also been demonstrated at greater densities, presumably due to aggregation artefacts. After density gradient centrifugation, the Rauscher mouse leukaemia virus also bands at a density of 1·15 (O'Connor, Rauscher and Zeigel, 1964). This relatively low density and the established sensitivity to ether of RLV would indicate the presence of an appreciable lipoprotein component in the virus.

Preliminary attempts have also been made to obtain infectious nucleic acid from RLV. Three nucleic acid extracts, prepared by the cold phenol method in the presence of bentonite and EDTA in phosphate buffer, pH 7·0, have exhibited leukaemogenic activity whereas two extracts (one hot, one cold, phenol) prepared in acetate buffer, pH 6·0, have been inert. These studies are continuing and will be fully reported elsewhere.

Efforts to visualize RLV by electron microscopy in radiogenic lymphomas of strain C57BL mice were unsuccessful (Dalton *et al.*, 1961). However, examination of the tissues of some of our C57BL mice, bearing lymphomas induced by serial-passage RLV preparations, revealed typical type-C particles in the

megakaryocytes of the bone marrow (Dalton, 1962). Similar observations have been made with serially passaged material from radiogenic lymphomas of C3H origin (Dmochowski, Grey and Gross, 1959). Recently we have collaborated with Professor William H. Carnes in electron micrographic studies of intrarenal thymus grafts, harvested at serial intervals after direct inoculation of RLV. These investigations, which are to be reported more fully elsewhere, have, to date, consistently revealed typical type-C particles budding from the plasma membrane of tumour cells and present in the intercellular spaces in early tumours developing in the thymus grafts within a few weeks after RLV injection. (Carnes *et al.*, 1966).

DIRECT INTERACTION OF VIRUS WITH THYMUS CELLS

Recent studies on the route of injection of RLV have yielded interesting results. Largely for reasons of convenience, we had utilized the intraperitoneal route of inoculation of newborn C57BL mice for the serial passage of RLV. However, when, confirming Law, Bradley and Rose (1963), a relatively low incidence of lymphomas was observed in thymectomized, irradiated C57BL and F_1 hybrid mice bearing intrarenal thymic grafts, the possibility was entertained that endogenous RLV is not efficiently delivered to the intrarenally grafted thymus. Accordingly, various routes of injection were compared. When RLV preparations (0·01 - 0·02 ml.) were injected directly into intrarenal thymus grafts in thymectomized, irradiated C57BL and (C57BL × AK)F_1 hosts, lymphomas consistently developed in the grafts with an incidence of 80 to 100 per cent and an average latent period of only 90 to 95 days (Haran-Ghera, Lieberman and Kaplan, 1966). The intravenous route of injection was almost equally effective in similarly prepared test animals but, since a larger volume of the RLV preparation (0·2 ml.) could be injected, direct comparison is not warranted. The somewhat lower yield

and longer latent period after intraperitoneal injection may be attributed to the more gradual delivery of virus to the circulation, with more efficient clearance in the renal glomerular effluent, or, perhaps, to phagocytosis by intraperitoneal macrophages with subsequent inactivation or sequestration of the virus.

Direct injection of RLV into one thymic lobe of intact, newborn C57BL mice has also proved highly effective in the induction of lymphomas. Tumours appeared microscopically within 20 to 30 days and were confined to the injected lobe initially (Haran-Ghera, Lieberman and Kaplan, 1966). These observations demonstrate that the leukaemogenic effect of RLV is exerted through a direct interaction with cells of the thymus.

PATHOGENESIS OF LYMPHOMAS AFTER DIRECT INJECTION OF VIRUS INTO INTRARENAL THYMUS GRAFTS

The pathogenesis of lymphomas arising in intrarenal thymus grafts has been studied by biopsy at serial intervals after direct injection of RLV. A portion of the graft was left undisturbed in the kidney and observed for ultimate tumour development. Part of the biopsy material was utilized for histological examination; the remainder was reimplanted under the kidney capsule in untreated, adult, C57BL host mice. Microtumours were recognizable histologically as early as one week after virus inoculation and gradually increased in number, extent, and invasiveness. There was a good general correlation between morphological and biological evidence of transplantability, as expressed by the development of neoplasms in the bioassay-implanted secondary host. Moreover, both of these indices correlated reasonably well with the tumour incidence expressed by the original intrarenal graft (Haran-Ghera, Lieberman and Kaplan, 1966). It would appear that potentially neoplastic cells develop in the graft in as little as one week after inoculation of RLV. However, the possibility that virus injected into the graft and carried over into

the secondary host gives rise to new virus-induced tumours has not been excluded. An experiment to distinguish between these two possibilities is now in progress, utilizing C57BL mice as the primary hosts and (C57BL × BALB) F_1 hybrids as the secondary hosts to permit determination of secondary-tumour transplantation behaviour.

Irradiation has been shown to induce alkaline-phosphatase activity in the mouse thymus, and elevated activity of this enzyme has been observed in lymphomas (Smith, 1961, 1962). Confirmatory observations were made in strain AKR lymphomas by Metcalf, Sparrow and Wyllie (1962). We have extended these studies in normal, irradiated, and thigh-shielded C57BL mice, with emphasis on the early stages in lymphoma development (Lagerlöf and Kaplan, 1965). The cortex of the normal C57BL thymus was histochemically negative for alkaline phosphatase. After irradiation, enzyme activity appeared concomitantly with, and limited geographically to, the earliest morphological signs of neoplastic change. After thigh-shielded irradiation, lymphomas were infrequent and enzyme activity remained correspondingly low. An association between morphological evidence of early tumour development and the appearance of alkaline-phosphatase activity was also demonstrable in RLV-induced lymphomas. Efforts to purify the enzyme are now in progress.

FACTORS INFLUENCING DONOR VERSUS HOST-CELL DERIVATION OF THYMUS-GRAFT LYMPHOMAS

In previous studies of lymphoma development in subcutaneous thymus implants of parental strain origin in thymectomized, irradiated F_1 hybrid recipients, it was established that the tumours were, in some instances, of donor origin and, in other instances, of host-cell origin (Kaplan, Hirsch and Brown, 1956; Law and Potter, 1956; Barnes *et al.*, 1959). The relative proportions of donor-to-host-cell derived tumours seemed to depend on the

genetic constitution of the F_1 hybrid used. We have now examined the transplantation behaviour of lymphomas arising in intrarenally implanted C57BL thymus grafts in (C57BL × AK)F_1 thymectomized, irradiated hybrid mice. When RLV injection into the graft was omitted, lymphomas developed in only 10 of 44 mice (23 per cent) with a mean latent period of 307 days. Seven of these 10 tumours were available for transplantation testing, and all 7 exhibited the hybrid (host-cell) type of transplantation behaviour. In contrast, when RLV was injected directly into C57BL thymus grafts in such prepared hybrid hosts, tumours developed in 13 of 15 mice (87 per cent) with a mean latent period of only 93 days, and 9 of the 12 tumours tested proved to be of C57BL donor type (Haran-Ghera, Lieberman and Kaplan, 1966). In a more recent experiment, the time interval between the implantation of the C57BL graft and the subsequent injection of RLV into the graft has been systematically varied (Lieberman and Kaplan, 1966). Virtually all of the tumours were of donor type when injection was made during the first two weeks after implantation, with a progressive shift toward neoplasms of host-cell derivation with increasing time thereafter.

LYMPHOMA DEVELOPMENT IN GRAFTS INJECTED WITH CELLS PREINCUBATED WITH VIRUS *in vitro*

Recently we have made an observation which further extends the usefulness of this intrarenal graft system (Lieberman and Kaplan, unpublished). Preparations of RLV have been incubated *in vitro* (1 hour at room temperature, then 2 to 3 hours in the cold) with suspensions of either adult peritoneal macrophages or thymocytes from newborn C57BL donors. The cells have then been centrifuged, washed repeatedly, and injected into thymus grafts in thymectomized, irradiated test animals. Lymphomas have developed in high incidence after a short latent period, as with the direct injection of RLV preparations into the intrarenal

graft. It has thus become possible to test the relative contributions of the epithelial and lymphocytic components of the thymus separately in an experiment in which thymectomized, irradiated F_1 hybrid hosts receive a thymus graft from one parent and then RLV-incubated thymocytes or macrophages from the other parent. That the viability of the grafted thymus epithelium is of some importance is indicated by an experiment in which thymuses from newborn C57BL donors were treated with a single dose of 5000 r., either just before or just after their removal from the donor, and immediately transplanted intrarenally. Even when such lethally irradiated grafts were subsequently injected with RLV, or with thymocytes preincubated in RLV preparations, virtually no lymphomas developed.

ASSOCIATION OF VIRUS WITH FRESHLY-INDUCED RADIOGENIC LYMPHOMAS

Although it may now be concluded that the leukaemogenic agent in the original extracts from our radiation-induced C57BL lymphomas was indeed a virus, it may be asked if it was merely a chance contaminant of the particular radiation-induced tumours tested at that time rather than a consistent and essential component of this induction system. By taking advantage of the potentiating effect of small doses of X-rays (Lieberman, Haran-Ghera and Kaplan, 1964) and the consistently high and early yield of lymphomas after direct injection of RLV into intrarenal thymus grafts (Haran-Ghera, Lieberman and Kaplan, 1966), it has been possible to increase the sensitivity of the bioassay of cell-free extracts for leukaemogenic activity sufficiently to permit investigation of this problem.

Cell-free extracts were prepared *individually* from each of a series of freshly induced C57BL radiogenic lymphomas, and tested by inoculation directly into intrarenal thymus grafts in thymectomized C57BL mice rendered susceptible by light whole-

body irradiation (2 doses of 100 r. one week apart). This experiment is still in progress. Our results so far show that lymphomas have developed in mice inoculated with each of the first 10 cell-free extracts tested and, in 7 of these, lymphoma incidence is appreciably greater than the base line response (about 7 per cent) attributable to the same dose of radiation alone. New serial passage lines established from some of these recent radiogenic viral extracts appear to have essentially the same properties as our long-established RLV preparations. It is concluded that RLV is consistently associated with, and extractable from, the radiation-induced lymphomas of strain C57BL mice. The most reasonable interpretation is that RLV is the agent actually responsible for the induction of lymphomas in irradiated C57BL mice and that radiation acts by inducing a change in the host–virus relationship. Cell-free extracts from mouse lymphomas induced by hydrocarbon carcinogens (Irino *et al.*, 1963; Toth, 1963) and nitroquinoline oxide (Kinosita, Tanaka and Kakefuda, 1964) have recently been shown to contain viral particles and/or to exhibit leukaemogenic activity. It seems likely that the conclusion which we have reached on the role of an occult virus in the induction of lymphomas in irradiated C57BL mice can be validly generalized to include many or all of the other external agents known to be leukaemogenic in the mouse.

ROLE OF RADIATION AND OTHER EXTERNAL CYTOTOXIC AGENTS IN THE LEUKAEMOGENIC PROCESS

Since the thymus is highly susceptible to lymphoma development at birth and since RLV is presumably present in newborn C57BL mice one wonders why mice of this strain do not spontaneously exhibit a high incidence of lymphomas. The most plausible explanation is that virus titre is low at the time of birth, perhaps because the virus is predominantly confined to other tissues which are not susceptible to its oncogenic action. Con-

sistent with this view is our finding that cell-free extracts of the thymus of newborn and young adult C57BL mice have little or no leukaemogenic activity. Later in life thymic involution occurs, and the thymus contains few of the immature lymphoblasts which appear to be the susceptible target cells for lymphoma induction (Kaplan and Brown, 1957; Kaplan, 1961; Axelrad and Van der Gaag, 1962). That virus titre ultimately rises is suggested by the appreciable leukaemogenic activity of tissue grafts from various organs of normal old C57BL mice (Rudali and Silberman, 1965).

What are the mechanisms by which radiation triggers the leukaemogenic potentialities of RLV? It has previously been demonstrated that thymus and bone-marrow injury are invariable concomitants of whole-body irradiation (Kaplan and Brown, 1957). Injury to these tissues is also produced by two other external leukaemogenic agents: urethane (Haran-Ghera and Kaplan, 1964; Fiore-Donati and Kaye, 1964) and methylcholanthrene (Yasuhira, 1964). Morphological studies (Kaplan and Brown, 1957; Kaplan, 1961) have revealed that, following radiation injury to the thymus, there is a period of regeneration during which an abundant population of immature lymphoid cells reappears. When the bone marrow is shielded, such cells are present only transiently and lymphocytic differentiation proceeds to completion; in contrast, when the bone marrow is irradiated, a maturation arrest is seen in the regenerating thymus and the susceptible lymphoblastic population is sustained at a high level.

In addition, radiation may cause the release of RLV from its natural *in vivo* habitat into the blood stream (Kaplan, 1964). The presence of a leukaemogenic agent in the plasma of irradiated C57BL mice has been reported (Mathé and Bernard, 1958). Experiments to test the plasma of irradiated C57BL mice for RLV viraemia are now in progress in our laboratory. Preliminary results, based on small numbers of animals, are suggestive of the existence of a transient viraemia 7 to 30 days after irradiation;

plasma from normal non-irradiated donors has exhibited no activity thus far.

However, even when the need for release of endogenous virus is obviated by the injection of exogenous RLV preparations into C57BL mice, small doses of radiation have been shown to potentiate the leukaemogenic activity of RLV to a striking degree (Lieberman, Haran-Ghera and Kaplan, 1964). The mechanism of this potentiation has not yet been elucidated but it differs in one important respect from the leukaemogenic action of radiation alone. The induction of thymic lymphomas in high incidence in strain C57BL mice requires whole-body X-ray exposure; irradiation with the bone marrow of the thigh (or the spleen) shielded profoundly inhibits lymphoma development. In striking contrast, however, thigh-shielded irradiation exhibits about the same degree of potentiation as whole-body irradiation when given in conjunction with exogenous RLV, suggesting that the potentiating mechanism does not involve radiation injury to the bone marrow. This potentiating effect on exogenous RLV leukaemogenesis is not limited to radiation; similar results have been obtained with two chemical agents which are leukaemogenic for this strain of mouse: urethane (Lieberman, Haran-Ghera and Kaplan, 1964) and thio-TEPA (Lieberman and Kaplan, unpublished). Although the doses of radiation required for potentiating action are small (2 or 3 exposures of 75 to 100 r.), the possibility that they may transiently depress immunological responsiveness and thus prevent inactivation of RLV *in vivo* requires investigation.

SUMMARY

Evidence is presented which establishes the viral nature of the leukaemogenic agent (RLV), observed some years ago in cell-free extracts from radiogenic thymic lymphomas of strain C57BL mice, and supports the view that RLV, rather than

radiation, is the real inducing agent for these neoplasms. Evidence for the existence of similar viruses in lymphomas induced by other external leukaemogenic agents has begun to emerge. It is suggested that mice harbouring such occult viruses may fail to exhibit a high spontaneous incidence of lymphoid tumours because the essential components of the induction system are out of phase: when the thymus is susceptible, virus titre is low and when virus titre rises, the thymus is no longer susceptible. It appears that whole-body irradiation succeeds in eliciting a high lymphoid-tumour response in mice of this strain by simultaneously releasing virus into the blood stream and by restoring to the adult thymus, as a secondary consequence of radiation injury to thymus and bone marrow, a sustained abundance of the immature lymphoid cells on which thymic susceptibility appears to depend.

Studies of the pathogenesis of lymphomas after direct injection of RLV into the thymus, when it is either *in situ* or grafted intrarenally, reveal morphological and biological evidence of neoplastic change in the first one to three weeks after injection; this results from a direct interaction between RLV and the thymus-lymphoid cell population. The earliest morphological indications of such lymphomas are accompanied by the apparently *de novo* appearance of histochemically demonstrable alkaline-phosphatase activity in the tumour cells. The proportion of tumours which are of donor *versus* host-cell origin is altered by the intrathymic injection of RLV and by the timing of such injection. Peritoneal macrophages or neonatal thymocytes preincubated with RLV *in vitro* can give rise to lymphomas after injection into intrarenally implanted thymus grafts.

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DISCUSSION

Clark: Dr. Kaplan, you concluded that the radiation leukaemia virus (RLV) and radiation itself injure the thymus. If this is their only effect how do you explain the failure of irradiated animals to recover after thymus tissue is transferred to them?

Kaplan: Dr. Miller showed in his presentation and we reported some years ago (Carnes, W. H., *et al.* [1956]. *Cancer Res.*, **16**, 429) that transplantation itself involves a massive injury to the thymus. Irradiation of the host has previously damaged the bone marrow so that the regeneration of this system also is interfered with. It is not, therefore, correct to assume that no injury is done to an irradiated animal by post-irradiation thymic grafting.

Clark: Do you think that this is simply an immunological phenomenon; that is, the virus is checked by an immunological mechanism, and, when this is damaged by irradiation, the virus is free to attack the thymus?

Kaplan: This certainly cannot be excluded but at this time we have no evidence either way.

Clark: Both you and Professor Furth have noted that the virus is present within the thymic epithelial cells. Is this the only place it is found?

Kaplan: No, that is only for the first ten days after irradiation, not later on. When a tumour is present, we see virtually nothing but tumour cells and the virus then appears at the plasma membrane of these cells.

Feldman: Dr. Kaplan, your experiments on the intracapsular transplantation of thymuses were carried out in thymectomized recipients. Have you done similar experiments in which the thymus of the recipient was retained and, if so, did the host thymus affect the neoplastic transformation within the grafted thymus?

Kaplan: Yes, we have done these experiments. We put an extra thymus graft into the kidney in intact animals under the same conditions and we found that the yield of tumours was the same, and there was exactly the same latent period, as in the thymectomized mice. Thus, we have no evidence that the presence of the animal's own thymus or any immunological competence which might have evolved from the thymus graft inhibit tumour formation. In our early experiments the thymuses were removed at weaning. We are now repeating this kind of study with neonatally thymectomized animals and I think this will be a more critical test of our hypothesis.

Feldman: Electron micrographs have demonstrated the virus particles first in the epithelial cells; it is only later that these particles seem to appear in the lymphoid cells. Do you think that this represents a true sequence of events, that is, does the virus first replicate in the epithelial component of the thymus and is it later transmitted to the lymphoid cells? Your other experiments indicated that a direct *in vitro* interaction between thymic lymphoid cells and virus may result in a leukaemogenic effect.

Kaplan: We obviously have a lot more to learn about this. When

the virus is injected directly into the thymus graft it may be that intermediaries, such as phagocytes or epithelial cells, are needed to produce a leukaemogenic effect. If the virus is incubated with C57BL thymic lymphocytes *in vitro*, these do become tumour cells but they seem to need the environment of the thymus to undergo transformation.

We have also injected virus directly into spleen and lymph node grafts in the kidney without producing tumours.

Ito: In the last three years we have done some work on urethane-induced thymic lymphoma in mice. (Ito, T., Hoshino, T., and Sawauchi, K. [1964]. *Z. Krebsforsch.*, **66,** 267; [1965]. *Z. Krebsforsch.* **66,** 552).

INCIDENCE OF NEOPLASTIC CHANGES IN THYMUSES OF MICE OF BOTH SEXES AT VARIOUS AGES

Age days)	*Sex*	*Number of mice*	*Non-neoplastic*		*Early neoplastic*		*Neoplastic*		*Neoplastic + early neoplastic*	
			Total	*Per cent*	*Total*	*Per cent*	*Total*	*Per cent*	*Total*	*Per cent*
50–60	M.	49	33	67·3	16	32·7	0	0	16	32·7
	F.	49	31	63·2	18	36·8	0	0	18	36·8
90	M.	43	18	41·8	22	51·1	3	7·1	25	58·2
	F.	45	12	26·7	30	66·6	3	6·7	33	73·3
100–150	M.	59	9	15·3	28	47·4	22	37·3	50	84·7
	F.	43	4	9·3	12	27·9	27	62·8	39	90·7

M = male. F = female.

The mice were of the Japanese dd strain—one of the most commonly used strains in Japan. This is a low-leukaemic strain and the incidence of spontaneous thymic lymphoma in it is extremely rare. Urethane was given repeatedly in early life as previously described by Ito *et al.* (1965, *loc. cit.*). Amongst the urethane-treated mice, after they were 100 days old, there gradually appeared mice which were moribund because of large thymic lymphomas. These animals were autopsied, and the rest of the mice were killed at 150 days and autopsied. The thymic lymphomas consisted histologically of neoplastic lymphocytes similar in appearance to immature large or medium lymphocytes. At 150 days, many of the thymuses although normal in weight and appearance showed neoplastic changes microscopically. The earliest changes

were replacement of the cortex by neoplastic lymphocytes and depletion of normal small lymphocytes. In some cases these changes were unilateral and in others bilateral. The incidence of the early neoplastic, non-neoplastic and neoplastic cases according to age is shown opposite. The earliest neoplastic changes, only visible microscopically, appeared early though infrequently in the latent period, and the incidence increased with advancing age. A sex difference in incidence was not seen early but appeared with age. At 90 days, macroscopic lymphomas were rare but early neoplastic changes were quite frequent. The total incidence of neoplastic and early neoplastic cases was higher in females than in males. In mice which were previously treated with urethane and then gonadectomized at 35 days, the incidence of neoplastic changes at 90 days was almost the same in both sexes as shown below:

INCIDENCE OF NEOPLASTIC CHANGES IN THYMUSES OF MICE GONADECTOMIZED AT 90 DAYS OF AGE

Sex	*Number of mice*	*Non-neoplastic*		*Early neoplastic*		*Neoplastic*		*Neoplastic + Early neoplastic*	
		Total	*Per cent*	*Total*	*Per cent*	*Total*	*Per cent*	*Total*	*Per cent*
M.	36	12	33·3	17	47·3	7	19·4	24	66·7
F.	35	11	31·4	16	45·9	8	22·6	24	68·5

M = male. F. = female.

The total incidence of thymomas (neoplastic plus early neoplastic cases) was slightly higher, and the incidence of neoplastic cases was also higher, in castrated males than in intact males. In ovariectomized females the total incidence of early neoplastic and neoplastic changes was slightly decreased, but the neoplastic changes alone were more common than in the intact females. The results indicate, in agreement with our earlier findings, that the testes exert an inhibiting action on the induction and growth of this lymphoma. It is difficult to draw conclusions about the action of the endogenous female sex hormones from these experiments. At 50–60 days, no tumour could be seen macroscopically and the incidence of early neoplastic cases was comparatively low and almost the same in both sexes. In adrenalectomized mice of the same age,

treated with urethane in early life and adrenalectomized nine or ten days before killing (at about 45–50 days), the incidence of neoplastic change was increased in both sexes and this increase was particularly marked in females.

Recently the physiological response of the thymus to adrenalectomy was reported to be different in the different sexes. A. L. Scherzer *et al.* (1963. *Archs Path.*, **76,** 647) noted that the main thymolytic role appears to be relegated to the adrenal glands in females and the testes in males. The present results suggest that a similar sex difference is seen in the effect of adrenalectomy on lymphoma development, as shown below:

INCIDENCE OF NEOPLASTIC CHANGES IN THYMUSES OF MICE ADRENALECTOMIZED AT 55–60 DAYS OF AGE

Sex	*Number of mice*	*Non-neoplastic*		*Early neoplastic*		*Neoplastic*	
		Total	*Per cent*	*Total*	*Per cent*	*Total*	*Per cent*
M.	37	20	54·0	17	46·0	0	0
F.	48	17	35·4	31	64·6	0	0

M = male. F. = female.

Metcalf: I would like to bring forward for discussion an alternative hypothesis for leukaemogenesis. In AKR mice infection with leukaemia virus occurs before birth but a very long latent period, averaging eight months, follows before leukaemia develops. This is very puzzling and in thinking about it I have been struck by the similarities between tolerance breakdown and leukaemogenesis. Thymectomy slows or prevents tolerance breakdown (Claman, H. W., and Talmage D. W. [1963]. *Science*, **141**, 1193) and thymus grafting accelerates it (Argyris, B. F. [1965]. *Transplantation*, **3,** 350). As Professor Furth has shown, thymectomy prevents leukaemogenesis whilst thymus grafting restores susceptibility to the development of leukaemia. Similarly, whole body irradiation accelerates tolerance breakdown (Fefer, A., and Nossal, G. J. V. [1962]. *Transplantatn Bull.*, **29,** 445) and is leukaemogenic (Kaplan, H.S. [1947]. *Cancer Res.*, **7,** 141).

The infection of animals with the Gross virus introduces a foreign antigen (either viral or viral-induced host antigen) into the animal—

the G-antigen (Old, L. J., Boyse, E. A., and Stockert, E. [1965]. *Cancer Res.*, **20**, 813). However, A. A. Axelrad (1965. *Prog. exp. Tumor Res.*, **6**, 31) has shown that AKR mice, because of their prenatal exposure to this antigen, are tolerant to it in exactly the same way that neonatally infected mice tolerate the lymphocytic choriomeningitis virus.

Is it possible that so long as tolerance persists no leukaemogenesis occurs, and that neoplastic change can only take place in infected lymphoid cells following breakdown in tolerance?

The AKR thymus exhibits puzzling changes in the preleukaemic period—there is development of medullary lymphoid follicles, plasma cells and germinal centres and evidence of an excessive destruction of small lymphocytes—all changes which might be expected following local intrathymic breakdown in tolerance to the G-antigen. If neoplastic change only occurred in virus-infected lymphocytes secondarily to an immune assault on these antigenic cells by other host cells, then thymectomy might be expected to prevent the development of leukaemia (a) by slowing or preventing tolerance breakdown or (b) by removing the local site of tolerance breakdown.

If this speculation is correct, it should be possible, using the battery of tests introduced by Professor Klein, to demonstrate the appearance of cells in the preleukaemic AKR thymus which could exert cytotoxic effects on other cells (such as cells from a neonatal AKR thymus) containing G-antigen. It is unlikely that circulating cytotoxic antibodies occur in preleukaemic mice, for we have found that neonatal AKR thymus grafts grow normally in preleukaemic AKR mice whose own thymuses exhibit evidence of cellular destruction (Metcalf, D., unpublished data).

Passaged leukaemia virus injected into older (competent) animals may induce leukaemia with a shorter latent period because the stage of immunological tolerance is bypassed in such animals.

Miller: Dr. Metcalf, how do you fit into your interesting scheme Rudali's finding that AKR leukaemic filtrates will accelerate AKR leukaemia when inoculated into newborn AKR mice (Rudali, G., Duplan, J.-F., and Latarjet, R. [1957]. *Bull. Ass. fr. Etude Cancer*, **44**, 440)? It would seem more likely, according to your hypothesis, that these filtrates would prolong tolerance so that leukaemia would either not develop or, at any rate, develop later rather than earlier.

Metcalf: There are many facts that are difficult to fit into this concept. I merely offer it as a new way of looking at the problem of leukaemogenesis. We seem to have reached a position of stalemate in our work with virus-induced leukaemias. There are still many unanswered questions about titres of virus at various times in the preleukaemic period and about the significance of cell proliferation and differentiation. If we could determine why the role of the thymus in tolerance breakdown and in leukaemogenesis is so remarkably similar we might make progress again in our analysis of leukaemogenesis.

Klein: Dr. Kaplan, you have shown that shielding the bone marrow, or infusion of bone-marrow cells, will protect against radiation-induced leukaemogenesis. What is the mechanism of this? Is this effect restricted to bone-marrow cells or can thymus and spleen cells protect in the same way? Finally, what cell type is responsible for this effect?

Kaplan: Spleen and bone marrow are the only tissues identified which have this effect of suppressing the development of leukaemia in the irradiated animal (Lorenz, E., Longdon, C. C., and Uphoff, D. [1953]. *J. natn. Cancer Inst.*, **44**, 291; Kaplan, H. S., Brown, M. B., and Paull, J. [1953]. *J. natn. Cancer Inst.*, **14**, 303). The cell type responsible for this is still a mystery, although the highly immature small lymphocyte-like cell is an intriguing candidate. We tried (Brown, M. B., *et al.* [1955]. *J. natn. Cancer Inst.*, **15**, 949) to find out if bone marrow, made erythroid or myeloid by pretreatment with phenylhydrazine or turpentine, differentially affected the response but such pretreatment did not seem to make any difference. These findings suggest that the more mature cell elements of the erythroid and myeloid series are not involved, but the results do not exclude the possibility that highly immature cells—stem cells perhaps—are responsible. Dr. Auerbach's comments on the possible existence of a humoral factor that comes from the bone marrow and influences thymic regeneration were very exciting because this confirms and extends some of our own early work (Berman, I., and Kaplan, H. S. unpublished data).

In irradiated animals one sees first injury and then regeneration of the thymus, but there is a remarkable difference between the regeneration patterns in animals irradiated with and without marrow or spleen shielding. In the unshielded animals there is a maturation arrest which

leaves the thymus abundantly supplied with large immature lymphoid cells for many days. In animals whose own bone marrow is shielded or animals into which compatible bone marrow has been injected, there is an extremely rapid phase of maturation during which these immature lymphoid cells persist for less than 24 hours. The answer to your question, then, may be that the virus has less time to contact susceptible target cells in the latter (protected) situation than in the former.

Auerbach: Strain differences may also be important. We have looked at the thymus at birth in a variety of strains (Auerbach, R. [1964]. The Thymus: Wistar Institute Symposium Monograph No. 2, p. 1, ed. Defendi, V., and Metcalf, D. Philadelphia: Wistar Institute Press). The AKR strain, judged by the Coulter counter, is slightly different from other strains we have tested and its thymus contains slightly larger cells for longer periods of time. After irradiation, even with low doses, there is a period of time when, although the thymus looks morphologically normal, the mean cell volume is slightly increased. This may explain why irradiation is needed to produce lymphatic leukaemia in the C57BL but not in the AKR strains.

Good: The classical way of eliminating immunological tolerance is by adaptive immunity. Volkert (Volkert, M., Larsen, J. H., and Pfau, C. J. [1964]. *Acta path. microbiol. scand.*, **61**, 268), working with the lymphatic choriomeningitis virus, showed that with preimmunized syngeneic cells, or even with non-immunized syngeneic cells, the lymphatic choriomeningitis infection can be eliminated or substantially reduced. Volkert (Spärck, J. V., and Volkert, M. [1965]. *Nature, Lond.*, **206**, 578) has also shown that Gross passage A virus can be eliminated during the incubation period of the leukaemia (by employing adoptive immunization with immunized syngeneic cells) without the animal developing leukaemia. We have been able to continue this work and it seems that this method of approach provides an extraordinary protection against leukaemia. These findings may be evidence against Dr. Metcalf's attractive hypothesis but they do not necessarily disprove it because the rate of elimination of the viral agent may be critical. It is possible that immunological deficiency is actually induced by the agents that give rise to the leukaemia. In one clinical syndrome in man —ataxia telangiectasia—there are both immunological deficiencies and an extraordinarily high incidence of lymphoma—eight or nine cases

out of approximately 125. The immunological deficiency in these cases is associated with the absence of γA-immunoglobulin in most cases and, occasionally, with deficiency of γG-immunoglobulin. These patients are not able to form antibodies as efficiently as normal people and their cellular immunity is deficient. In four cases we have found an abnormal thymus reminiscent of the embryonic thymus at the stage of earliest lymphoid development. In congenital, sex-linked, recessive agammaglobulinaemia, also, there is a high incidence of acute leukaemia. In Chediak-Higashi disease there is a completely different deficiency: the γ-globulins and all other antibody-producing capacities are normal, but there is an abnormality of the leucocyte granules; indeed, all the lysosomes of the body may be abnormal. These patients are susceptible to bacterial and other infections, but if they live long enough nearly all of them die from lymphosarcoma. I have seen this in four, and there have been at least a dozen such recorded, cases. This is a form of cancer in man in which association with a virus can be demonstrated. Dr. James White, studying blood from our patients by electron microscopy, has shown distinct virus particles of the helical type. There are large numbers of these particles, at least 10,000–20,000 particles in each infected cell.

Metcalf: We have taken preleukaemic AKR spleen cells of the tolerance-breaking type and injected them into young adult (AKR$\times$C3H)F_1 mice, and have shown that they seed into the thymus (Metcalf, D. [1963]. *Cancer Res.*, **23**, 1774). The injected cells themselves become neoplastic and the onset of other neoplasms in the host is accelerated by the injection. We have found, contrary to Professor Good's comments on the immunological unresponsiveness of preleukaemic mice, that AKR mice, at least, are exceptionally good producers of haemagglutinins to sheep red blood cells. This suggests that preleukaemic mice certainly do not have a generalized depression of immune responses (Metcalf, D., unpublished data).

Good: I do not want to be misunderstood. I was not suggesting that every mouse that develops leukaemia is completely incompetent immunologically. They may be immunologically deficient to one particular agent or have a moderate generalized immunological deficiency. It is known from the work of Prehn, R. T. (1963. *J. natn. Cancer Inst.*, **31**, 791) that most, if not all, carcinogenic agents are not

only carcinogenic, but immunosuppressive and carcinostatic as well. This, I believe, is a most important relationship.

Auerbach: It seems very exciting to me that if the thymus is affected early a donor-type of leukaemia develops. Dr. Kaplan, have you looked at the non-leukaemic cells of the grafted thymus to see if there is the same effect on the uninfected cells, or is this persistence in survival limited to virus-infected cells?

Kaplan: There is really no appropriate way to do this because the tumour cells so quickly take over the parent graft. By about four weeks there is virtually complete replacement of the graft by tumour cells.

Furth: There is a complex relationship between immunological deficiency and the incidence of neoplasia. Inhibition of antibody production promotes replication of the virus and increases viraemia with consequent induction of neoplasia, but it probably also induces antigenic modifications. Modified cells may be suppressed in normal hosts but not in immunologically incompetent hosts. Most murine leukaemias are unique as the thymus is necessary for their induction. Thymectomy, or just thymic involution, inhibits leukaemia induction. Some leukaemias do not arise in the thymus, and therefore immunological deficiency (best induced by neonatal thymectomy) may enhance their induction as it enhances the induction of neoplasms in general. Nutritional disturbances, common in thymectomized hosts, act in the opposite direction. Irradiation may act as both a transient immunodepressant and permanent modifier of many cells.

In electron micrographs, few replicating virus particles are seen inside the plasma membranes of the cells, but complete virus is present in between the cells in large numbers. It is puzzling how such a virus can give rise to neoplastic transformation.

Kaplan: I do not agree that the presence of virus at the plasma membrane is a puzzling phenomenon. If the virus genome really determines the abnormal behaviour of the cell on which it is parasitic then its position in the cell is really irrelevant. Most RNA viruses can utilize, for the synthesis of new viral RNA, metabolic machinery which does not involve the DNA-dependent polymerases of the nucleus. Therefore, I find nothing paradoxical about the presence of the virus at the cell membrane. I *am* intrigued that this particular neoplasm

so regularly yields the alkaline–phosphatase reaction. We have investigated artificial involution and hyperplasia of the thymus; in neither of these states is there alkaline-phosphatase activity in normal thymic lymphocytes (Lagerlöf, B., and Kaplan, H. S. [1965]. *Fedn Proc. Fedn Am. Socs exp. Biol.*, **24**, 684 [abstract]). These findings suggest that the virus may be inducing this enzyme *de novo* in the tumour cells. Purification of the virus and very careful immunological studies must be done before firm conclusions can be reached on this important point.

MAST CELLS IN THE MOUSE THYMUS

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For several years I have been deeply interested in the histology of the thymus in NZB mice and their hybrids and, in particular, in the appearance of the proliferative medullary lesions that occur from four to six months of age onward. It was a very early observation that mast cells were much more common in NZB thymuses than in other mouse strains and there was a pronounced concentration of mast cells in the cortex adjacent to the cortico-medullary junction. This concentration was even more evident in the vicinity of an expanded area of medulla about a developing proliferative lesion.

Then in one week I saw two spectacular examples of massive mast-cell change occupying a considerable area of a thymus section. These thymuses have been described (Burnet, 1965) and other examples have now been discovered. The total is now nine examples from about 1500 thymuses examined, plus one example of similar change in a mesenteric lymph node. Fairly extensive experiments to produce the condition deliberately have been inconclusive. It may be significant, however, that almost all the examples have been found in mice undergoing experiments which involve some additional immunological stress. Two were found in functioning thymus grafts and two more in animals carrying thymus grafts. One each came from mice treated by local irradiation of the thymus, heparin treatment and Imuran treatment. Only one came from a mouse killed when moribund in the large number of "natural history" studies. None of this

may have any special relevance until we know how long it takes for a substantial area of change to develop in the thymus, and, once developed, what are the circumstances that lead to its disappearance or indefinite continuation?

MASSIVE MAST-CELL METAPLASIA

A full account of the appearances of this condition has been published (Burnet, 1965), and here I shall merely summarize the deductions which I believe can be drawn from the histological appearances. I regret, exceedingly, that we have failed to find a way of reproducing the massive mast-cell change experimentally. The more important deductions are:

(*a*) The mast cells arise by a change *in situ* of pre-existent thymocytes. The distribution and the complete absence of mitosis in the mast cells make any other origin inconceivable. Appearances showing very light granulation, especially in the perinuclear region, can be interpreted as the change in progress.

(*b*) The change avoids medullary areas but in at least two instances appeared to start in a germinal centre in the medulla. The process seems to involve initially cells capable of proliferation and perhaps in the process of proliferation.

(*c*) Lymphocytes only are involved initially; epithelial cells and macrophages are unaffected. As the process develops there is a gradual disappearance of these other cell types, presumably by a process of pressure autolysis and proteolysis by enzymes from the mast cells. Eventually, a uniform mass of mast cells may cover the whole area of the section.

(*d*) There is evidence to suggest that the change at first involves the cells of a single "package" (Clark, 1963).

(*e*) Everything points to the centrifugal spread of a transforming influence from the primary site of transformation. This

influence can hardly be actual movement of cells and the only reasonable alternative is that the process of lymphocyte-to-mast-cell change results in the liberation of diffusible material which, in its turn, can induce the change in adjacent lymphocytes.

(*f*) Since the marginal areas are made up of *discrete* patches of changed cells, it follows that cells must differ in their primary receptiveness to the stimulus.

CELLULAR CHANGES IN THE NZB THYMUS

In the thymuses of most breeds of pure-line mice there are usually very few mast cells. These are confined to the connective tissue of the capsule with an occasional one along a major vessel dipping into the substance of the thymus. These mast cells are heavily granulated and are typically slug-like in profile. They can readily be seen in NZB thymuses but differ sharply from the type of mast cell which arises in the substance of the cortex and with which we are concerned. The cortical mast cells are more polygonal in outline and with more lightly staining granules. With the aldehyde fuchsin stain, demonstrated to us by Dr. D. W. Menzies, both forms of mast cell are metachromatic but the peripheral ones stain greenish purple, the cortical ones red.

At the margin of an expanding medullary area within which proliferative processes are at work there are varying concentrations of mast cells, more often in small groups than in isolation. Mast cells are, however, only the most conspicuous of a number of other aberrant types of cell seen in the expanding area. A wide variety of relatively fine, textured mosaics of cells can be seen. These include, in addition to the thymocyte and the reticulo-epithelial cell, macrophages with a characteristic foamy appearance which stain brightly with periodic acid-Schiff (PAS), pyroninophilic cells approaching plasma-cell character, mast cells

and small round cells with paler nuclei than those of "normal" lymphocytes.

In general, sections stained satisfactorily with Unna-Pappenheim stain show a pinkish rim at the margin of the expanding medulla due to the accumulation of pyroninophilic cells, foamy cells and mast cells. This raises the question of the details of the cytological processes associated with a relatively rapid expansion

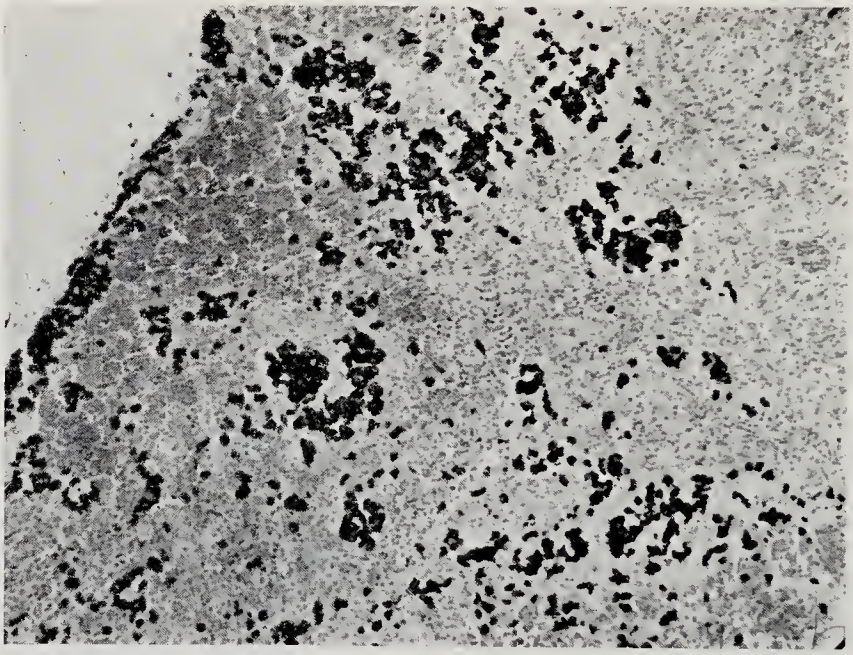

FIG. 1. A typical mild example of mast-cell change in the thymus of an AKR × NZB hybrid showing distribution of mast cells in characteristic clumps with a concentration immediately beneath the capsule of the thymus. Toluidine Blue. (× 100).

of the medulla. The distribution of plasma cells has been taken throughout as an index of the changes and, indeed, as an indication that change is taking place *in situ*. The first point to be stressed is that in Unna-Pappenheim-stained sections there are often cells that appear to be intermediate between pyroninophilic cells of plasmablast type and mast cells. Even more frequently one finds

in the same clump of cells pyroninophilic cells, mast cells and pale round cells. Where the medulla is expanding rapidly, particularly in (NZB × NZW)F_1 thymuses, one sees a moth-eaten appearance in front and fairly diffuse clumps of plasma cells in the recently expanded zones. Also, in (NZB × NZW)F_1 mice, one may see almost massive conversion of a large area of cortex to pyroninophilic cells approaching plasma cells in character.

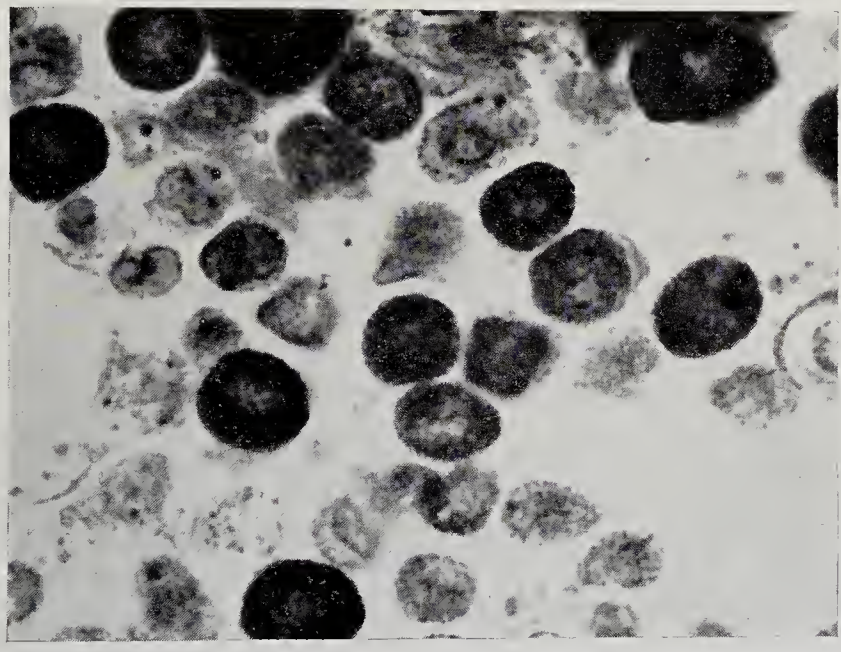

FIG. 2. Mast cells showing widely varying intensity of granulation in an area of massive mast-cell change. Thymus AKR × NZB hybrid 123. Unna Pappenheim. (× 1300).

Our current interpretation of these appearances is that the thymocytes are in a labile state in the process of their differentiation from stem cells to immunocytes. In that state they are also highly susceptible to various agents (known and unknown) which can stimulate them to differentiation or to self-destruction. It would be in line with current immunological thought for the simplest change to be to a pyroninophilic cell with increased

cytoplasm and that from this a variety of secondary developments, depending on the local micro-environment, could follow to plasma cell, mast cell or reversion to lymphocyte. At the same time the reticulo-epithelial cells are also subject to change in this process. Quick elimination of lymphocytes by X-irradiation or cytotoxic drugs may leave the cortex virtually empty of all but epithelial cells and proliferation of epithelial cells around a small blood vessel is often seen. Current opinion is that the PAS-positive and foamy cells are derived from the epithelium which is itself phagocytic. It is reasonable to believe that under stresses leading to anomalous differentiation of lymphocytes, anomalies of reticulo-epithelial development will also arise.

CONCLUSION

This point of view does not make much contact with current thought either on thymic function or on general immunological theory but I hope that it may eventually lead to a little more understanding of the process of differentiation. At least the pictures of massive metaplasia of mast cells that I have shown are rather startling indications of differentiation directed by some agent potentially capable of experimental study. And I believe that it would not be out of line with the history of experimental biology to predict that in ten years' time theoretical immunology, as we know it now, may have withered on the bough and been overgrown by a vigorous experimental approach to the understanding of the broader subject of differentiation and morphogenesis.

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DISCUSSION

Gowans: H. Ginsburg and L. Sachs (1963. *J. natn. Cancer Inst.*, **31**, 1) have published observations which are very similar to those just described in the thymus of NZB mice by Sir Macfarlane. A suspension of thymus cells was cultivated *in vitro* on the surface of a feeder layer of embryo cells and, after a period of incubation, the whole culture became overgrown with a most impressive growth of mast cells. It is not clear if these were derived from thymic lymphocytes, or by cell division from some other precursor but they certainly seemed to take origin from something in the thymus.

Burnet: In Dr. Feldman's laboratory a similar picture emerged in which the cells changed gradually in the course of multiplication. Isn't this right Dr. Feldman?

Feldman: Yes. Experiments carried out by Dr. H. Ginsburg of the Weizmann Institute have demonstrated the *in vitro* differentiation of mast cells from normal mouse thymus cells (Ginsburg, H. [1963]. *Ann. N.Y. Acad. Sci.*, **103**, 20). In more recent experiments mast-cell differentiation has been obtained *in vitro* from spleen cells; mast-cell formation, at least in cell cultures, is not restricted to the thymus.

Burnet: These findings do suggest with reasonable certainty that thymic lymphocytes do change into mast cells.

Clark: E. D. Korn (1959. *J. biol. Chem.*, **234**, 1321) has shown that mast cells manufacture their own heparin. It has also been suspected by J. F. Riley (1959. The Mast Cells. London: Livingstone) that mast cells may take part in an exchange of acid mucopolysaccharides with the ground substance of connective tissue. The changes that occur *in vitro* over a feeder layer of mast cells also suggest that lymphocytes can become mast cells by the uptake of acid mucopolysaccharide from the feeder layer. I wonder, therefore, if some miscarriage of acid-mucopolysaccharide metabolism in thymic epithelial cells may play a role in the transformation of thymic lymphocytes into mast cells.

Burnet: Occasionally I have thought that I have seen mast-cell change taking place in epithelial cells, but usually this does not happen.

Clark: I did not intend to imply that the epithelial cells became mast cells, but that they might be a source of acid mucopolysaccharide.

Burnet: An enormous amount of mucopolysaccharide would have to be made to produce this picture.

Metcalf: Ginsburg and Sachs (1963, *loc. cit.*) recorded that when the Moloney virus was added to their *in vitro* thymocyte-mast-cell system the transformation from thymocyte to mast cell occurred more frequently than when the virus was not present. This observation is relevant to Sir Macfarlane's comment that the thymus in the (AKR× NZB) F_1 hybrid is particularly prone to show mast-cell change. Dr. Feldman, do you think that the Gross virus these mice carry might influence the susceptibility of (AKR×NZB) F_1 thymic cells to transformation into mast cells?

Feldman: I do not know about this. In culture one can obtain transformation into mast cells without the assistance of any viruses that we know of.

Burnet: My reason for commenting on the change of epithelial cells to mast cells was just that I found two consecutive slides from (AKR× NZB) hybrids showing the mast-cell change, but it may be no commoner in these hybrids than in the NZB mice.

Auerbach: Do changes in the tissue organization of the thymus, or of connective tissue in general, precede the appearance of the mast cells? In tissue culture, at least, transformation of this type results from previous morphological changes which seem to invite the shift to mast cells.

Burnet: This may be so. Mast-cell counts in the thymuses of NZB mice at various ages show that there are practically no mast cells in the very young thymus, that is, at three, four, five and six weeks. After this, the mast-cell count increases steadily up to around four to five months. There is no particular relationship between the mast-cell count and the development of the characteristic lesions which appear at five, six and seven months, but the examples with very numerous mast cells are usually in the older thymuses in which damage of an auto-immune type has already occurred. I have only found these high mast-cell counts in thymuses with definite evidence of immunological pathology.

Good: It has now been claimed many times that cells can turn from one sort into another. But is this really true? Are we neglecting the possibility of associated but independent proliferative phenomena?

Sir Macfarlane, have you studied the uptake of tritiated thymidine in this context? Is there really evidence that lymphocytes transform to mast cells or are the blast cells of the mast-cell line lymphocyte-like? If proliferation of the mast-cell line were favoured, this could easily look like transformation from lymphocyte to mast cell.

Burnet: I have not done tritiated-thymidine uptake studies, but the complete absence of mitotic figures over enormous sheets of these mast cells is strong evidence against a proliferative reaction and, therefore, in favour of transformation.

Good: Your argument against a proliferative reaction may be misleading. Roberts and Dixon (Roberts, J. C., Jr., and Dixon, F. J. [1955]. *J. exp. Med.*, **102**, 379), too, were convinced that mononuclear lymphocyte-like cells were transformed into plasma cells but, in retrospect, it seems much more likely that mitotic processes were present in their cultures, but in only a few cells at a time, and Roberts and Dixon were just not seeing the mitotic figures.

Dameshek: I agree with Professor Good. After all, even if there are no mitoses, these must be masses of mast-cell tumours.

Burnet: I disagree with you. They are big groups of cells, certainly, but there are no mitoses.

Dameshek: The development of big groups of mast cells is reminiscent of the development of lymphomatous masses in a proliferation that is essentially immunological, that is, in graft-versus-host disease following the injection of parental spleen cells into F_1 hybrids. What is at first a self-perpetuating auto-immune reaction may eventually become leukaemic, as in NZB mice. Could what Sir Macfarlane has described be a neoplastic type of reaction that starts as an immunological one? (Mellors, R. C. [1966]. *Blood*, **27**, 435). There may be a clinical analogue of this lymphocyte-mast-cell relationship since it occurs not infrequently in the bone marrow of cases of macroglobulinaemia which may show not only a striking lymphocytosis but also a marked mastocytosis. Macroglobulinaemia itself may be an aberrant form of immunological disease.

Burnet: There is a good deal of evidence against the view that these mast cells are neoplastic. We have looked rather hard for metastases without finding any and, very characteristically, the process that I have described is a local one present in only one lobe of the thymus.

Dameshek: An example in clinical practice of a localized lesion which becomes proliferative and neoplastic is the mastocytoma of urticaria pigmentosa. This may perisist for years but eventually it becomes generalized and, in some cases, produces the so-called mast-cell leukaemia. I suppose one cannot say unequivocally that your sheets of mast cells will not some day also become generalized and metastasize to other sites.

Nossal: Sir Macfarlane, in the earliest beginnings of these proliferations have you noticed any arteriolar hyalinosis or any of the other arterial changes described by Hicks (Hicks, J. D. [1966]. *J. Path. Bact.*, in press)? I am reminded of some unpublished studies that Szenberg and I were doing on antibody production *in vitro* using lymph node fragments. In these we encountered a massive development of mast cells but, at that time, our fragments did not form antibodies as well as we had hoped and we discarded them. Then, after your experiments, we tried to repeat our own but by this time our tissue culture methods had improved—presumably oxygenation and nutrition of the tissues were better—and our lymph node fragments produced no mast cells and plenty of antibody! Do you think that the trigger, at least in part, for the mast cell change could be deficient oxygenation due to past arteriolar damage?

Burnet: We could take this point further and speculate that the deficient oxygenation is due to the close packing of immunologically proliferating cells. What evidence I do have suggests that the transformation process starts in these proliferating regions of immunologically stimulated cells. There are all sorts of useful experiments I could do if I knew how to initiate this transformation process; unfortunately I only know that it exists when I see it in a paraffin section.

I have one unique slide which shows mast cells without granules. In this section, from one particular thymus, there is an extraordinary gradient of cells. The cells, stained with haematoxylin and eosin, appear to be of a uniform and unusual type. They have the general characteristics of the mast cell, but the central cells in the section have no granules whereas the peripheral ones are typical mast cells with granules.

Dameshek: Can they be transplanted?

Burnet: We cannot do transplants or any of these other experiments

until we can spot this material before it reaches the paraffin block, and this we have not yet been able to do, have we Dr. Holmes?

Holmes: No, not yet.

Furth: The observation on the transformation of thymic lymphocytes to mast cells surprises me. We have studied mast cells, both normal and neoplastic types, extensively. We have found mast cells distributed throughout the body in many organs, and characteristically in certain tumours, but not specifically associated with lymphocytes. We have been particularly impressed by the lack of mast cells in the bone marrow. The origin of the mast cell remains a mystery to us and we have concluded that mast cells come from mast cells.

What initiated our survey of the mast cell was the study of a benign transplantable splenic tumour in AK mice (Bali, T., and Furth, J. [1949]. *Am. J. Path.*, **20**, 605). This tumour was composed of cells resembling epithelial-reticular cells and was characteristically rich in mast cells. Later it was found that progesterone-secreting luteomas also contained many mast cells while oestrogen-secreting granulosa-cell tumours did not. Histogenetically the mast cells did not seem to be related to these neoplastic cells. We have studied for many years a transplantable mastocytoma and so it is unlikely that we have missed mast cells in murine thymuses. The presence of mast cells in the thymuses of NZB mice reminds me of the abundance of these cells in splenic epthelial-reticulum-cell tumours.

Professor Dameshek has suggested that many benign-looking tumours take a long time to evolve. When such tumours are transplanted successively they grow more rapidly and may ultimately become malignant. The fact that we rarely see mitoses does not exclude the possibility that the cells we are observing multiply by mitosis.

Burnet: I am sure they must divide by mitosis to be there at all. Most mast cells seen in the tissues have a characteristic appearance. They are elongated cells and look precisely the same as the sort of mast cell that we see normally on the capsule of the thymus. Peritoneal tissue, also, always contains a few mast cells. But these "normal" mast cells have slightly different staining qualities from the ones I am concerned with which are seen in the middle of the cortex of the thymus. Dr. Metcalf, what staining method did we use to differentiate between these two types of mast cells?

Metcalf: We used a stain for elastin to which the mast cells of the connective tissues react positively by staining purple. The granules of mast cells from the thymic cortex, and some others that we were interested in from the spleen, gave a positive red stain for elastin, and elastin fibres often seemed to enter the cells and become contiguous with the mast-cell granules.

Furth: Do the mast cells in NZB mice contain the three characteristic hormones—heparin, serotonin and histamine—of the ordinary mast cells?

Burnet: We simply do not have enough material to answer that question. We only find these special cells in about 1 per cent of the thymuses we examine. A major problem here is to find a way to detect the cells before the sections are fixed.

Rowley: Does the $(AKR \times NZB)F_1$ hybrid lack the same complement component that is lacking in the pure strain of NZB mice?

Holmes: There is some doubt if AKR mice possess this complement antigen; reports from different workers about AKR mice possessing the complement antigen have been conflicting (Cinader, B., Dubiski, S., and Wardlaw, A. C. [1964]. *J. exp. Med.*, **120**, 897; Erickson, R. P., Tachibana, D. K., Herzenberg, L. A., and Herzenberg, L. T. [1964]. *J. Immun.*, **92**, 611).

Burnet: Certain other hybrids do possess the complement antigen. C57BL mice do, and so there is presumably one such component in the $(C57BL \times NZB)F_1$ hybrid. As this behaves like the $(AKR \times NZB)F_1$ hybrid, I presume that the answer to this question is that there *is* a complement fraction in the latter hybrid.

Rowley: What is the effect of thymectomy on complement?

Burnet: I do not know.

Good: Dr. Gewurz in my laboratory has investigated this question in our agammaglobulinaemic patients and has found that the thymus does not have a significant effect on the complement system. In the Swiss type of agammaglobulinaemia, for instance, all components of complement are present and, although some depression of the first component was seen, the total complement levels were normal and the fourth component was increased.

Strauss: We know that degranulation of mast cells takes place in the presence of antigen-antibody complexes. Sir Macfarlane, do you think

that the appearance of the agranular mast cells you have described in the germinal follicles of the thymus of the NZB mouse may be an integral part of the process that we call auto-immunity?

Burnet: These mast cells without granules are very rare. I would stress that massive mast-cell change was only seen in 0·2–0·5 per cent of the thymuses I have examined.

Kaplan: In view of the connexion of mast cells with histamine do you think that prolonged treatment with histamine-releasing substances such as 48/80 would increase the yield of these lesions in your mice and give you greater supplies of material to work with?

Burnet: We have not used a histamine-releasing substance but we have tried a number of other things—cytotoxic drugs, local irradiation and injections of heparin—without really producing more of these lesions. Dr. Diener has obtained only an occasional positive result which may have had no relation to the manipulations he used.

Auerbach: Csaba and his colleagues in Hungary have written on the distribution of mast cells (Csaba, G., *et al.* [1960]. *Acta morph. hung.*, **9**, 285; Csaba, G., Törö, I., and Bodoky, M., [1963]. *Z. mikrosk.-anat. Forsch.*, **70**, 242). Sir Macfarlane, do you know if they used a peculiar strain of mice, perhaps even a diseased strain?

Burnet: Those papers were not referring to the phenomenon I have been describing.

Dameshek: We should all thank Sir Macfarlane for bringing the mast cell again to our notice. We know little more about its function now than was known when Ehrlich first named it "mast cell" or the "cell of nutrition". It is still not certain whether it is derived from the reticulo-endothelial cells, as we used to think, or from the lymphocytes, more specifically from the thymic lymphocytes.

Burnet: There may be more than one mast cell.

Dameshek: Lately, I have wanted to include it among the blood cells and it may be that your presentation and comments have added evidence to support this inclination.

LYMPHOCYTES AND ANTIBODIES IN RELATION TO MALIGNANT DISEASE†

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The purpose of this paper was to discuss the various rejection mechanisms that animal hosts can direct against antigenic tumour cells. In all such discussions it is essential to distinguish between ordinary transplantation antigens and tumour-specific antigens. It seems useful to consider first, as a model, the ways in which an animal is known to deal with tumour cells that carry foreign, "strong" transplantation antigens and then to proceed to examine the tumour-specific antigens. There are two reasons for this: much more is known about the rejection reactions directed against transplantation antigens of the H-2 type in mice than about any other single system; and, there are good reasons to assume that rejection mechanisms in the host are identical in principle for the different antigenic systems even though there may be considerable variation in detail, depending on the strength of the antigenic barrier.

HOST REACTIONS AGAINST TUMOUR CELLS CARRYING FOREIGN TRANSPLANTATION ANTIGENS

These can be classified into *humoral* and *cell-bound* mechanisms. Both have been studied most extensively with the H-2 system in mice. For humoral mechanisms, different cell types have been

† Shortened version of an open lecture. The entire presentation is published elsewhere (Klein, G. [1966]. *In* Viruses Inducing Cancer, Implications for Therapy, p.323, ed. Burdette, W. J., Salt Lake City: University of Utah Press).

shown to vary considerably in their sensitivity to the cytoxic action of anti-H-2 isoantisera in the presence of complement. It has been shown by Möller and Möller (1962) that the antigenic surface receptor density is the critical variable: cytotoxic sensitivity results if the density exceeds a certain level and resistance if it is below this level. A direct relationship could be demonstrated between the ability of different cell types to absorb anti-H-2 antibodies, calculated per unit cell surface, and their cytotoxic sensitivity. Furthermore, the cytotoxic sensitivity of a given type of target cell could be increased experimentally by extending the number of reactive antigenic sites on it. This could be done by combining two or three antisera directed against different individual components of the H-2 system.

The mechanism by which sensitized host cells kill appropriate target cells is not clear but recent work in several laboratories has strongly suggested that, while the adherence of sensitized host cells to the target cells is probably mediated by antibody, the killing itself is primarily due to other factors. Work in our laboratory has shown that great significance must be attributed to the close contact established between lymphocytes and target cells carrying different *antigenic* configurations on their cell surface (Hellström, 1964; Möller, 1965*a*; Hellström and Hellström, 1965). In these circumstances non-immune allogeneic lymphocytes are as efficient as immune cells. It appeared possible that the non-immune allogeneic lymphocytes from the lymph node became immunized as a result of their direct contact with the antigenically-foreign target cells, even though this seemed unlikely because of the relatively short periods involved (24–48 hours). This possibility became quite untenable, however, when it was found that F_1 lymph node cells, semi-syngeneic to the target cells, were as efficient as allogeneic cells after phytohaemagglutinin treatment in killing the target cells. F_1 cells of this kind, according to the available evidence, are not immunologically competent to react against target cells derived from one

parental strain. It follows from this that the mechanism governing the killing action of these semi-allogeneic, semi-syngeneic cells is not an immunological one. It was assumed, therefore, that a close contact between cells carrying different H-2 specificities is, *per se*, sufficient to induce a cytotoxic reaction. It must be emphasized that this type of non-immunological killing has so far only been demonstrated with the H-2 system and it is not known if the effect is limited to this system or if it may have a more general significance. It is not known exactly how the killing occurs when allogeneic cells are brought closely together. The non-immunological nature of the mechanism is also strongly suggested by the most recent finding of Hellström (to be published) and of Möller (1966) that X-irradiated cells can exert this cytotoxic effect and that it is not restricted to lymphocytes but even liver cells and neoplastic cells can function as effectors. This suggests that the immune elimination of cells with foreign antigenic sites may represent a special case within the framework of a more general mechanism. This may have been established by evolution, due to the mechanism's potentiality for eliminating somatic cell mutants with changed surface components. Development of the antibody forming system would be merely a superimposed consequence adapted to permit a mobile cell, the lymphocyte, to search out the antigenic target by its differentiated function—antibody production—but to kill it by the same mechanism that is already available locally for other tissue cells.

Killing that is mediated through cells has been shown to be effective *in vivo* with all systems where transplant rejection reactions could be found in ordinary grafting tests. This applies to strong transplantation antigens of the H-2 type, weak transplantation antigens of non-H-2 types and to tumour-specific antigen systems as well. All target cell types tested were sensitive to the inhibitory effect of lymphoid cells or macrophages, even though the optimal ratio between sensitized lymph node cells and target cells needed for a clear inhibitory effect has varied,

depending on the system and various technical details concerning materials and methods. Cell killing effects *in vitro* have so far been mainly restricted to the H-2 system.

TUMOUR CELL ESCAPE FROM HOST REJECTION REACTIONS: ANTIGENIC LOSS VERSUS IMMUNORESISTANCE

Two basically different kinds of mechanisms have been demonstrated by which tumour cells may circumvent rejection in the H-2 system. These mechanisms can be classified as antigenic losses, and development of immunoresistance without complete antigenic loss. In the former group, permanent complete and irreversible losses of entire H-2 complexes have been demonstrated in one situation only. Tumours induced in F_1 hybrid hosts xenogeneic for the H-2 locus and thus carrying two H-2 complexes of distinctly different specificities were transplanted to the parental strains and thus exposed to a strong selection pressure directed against one antigenic complex at a time. Variant tumour sublines could be isolated from a large number of different F_1 hybrid tumours. These sublines were specifically compatible with one parental strain having lost the entire H-2 complex of the opposite parental strain as shown by a number of independent tests (Klein, Klein and Revesz, 1957; Klein, Klein and Hellström, 1960). The loss of one H-2 complex is an irreversible change: prolonged serial passage through the F_1 hybrid in which the tumour was originally induced does not lead to the reappearance of the missing antigenic phenotype. In all probability this loss is mediated by a chromosomal mechanism—possibly somatic crossing over (Klein and Klein, 1964). It has never been shown that tumour cells that have only one antigenic complex (either because they are syngeneic or because they have lost the other) can lose this single complex. When these cells are subjected to immunoselection, however, the antigen concentration per cell often decreases considerably, leading to immunoresistance and the so-called

non-specific growth that violates ordinary transplantation barriers (Möller, 1964). Such tumours still carry the relevant H-2 antigenic receptors but are not killed by the action by humoral antibody. They are particularly susceptible to enhancement and may often show a phenomenon of "self-enhancement" in the course of their growth in a foreign host. They induce humoral antibodies which coat the foreign antigenic sites on the surface of the tumour cell and thereby protect the target from the more efficient rejection mediated by host cells. Coating of the target cell has been shown to prevent killing by immune lymphocytes both *in vitro* and *in vivo* (Möller, G., 1963; Möller, E., 1965*b*).

TUMOUR SPECIFIC ANTIGENS

Specific antigenicity capable of eliciting resistance in genetically compatible (syngeneic) animals has been demonstrated in all chemically and virally induced tumours so far studied (Sjögren, 1965). The complete cross-reactivity of neoplasms induced by the same virus, even if they are of different morphology, is in striking contrast to the individually specific antigenicity of chemically induced tumours which are often of very similar morphology. Although tumour-specific antigens vary in strength in individual tumours, these antigens are comparable to weak histocompatability antigens.

Host defence mechanisms directed against tumour-specific transplantation antigens can also be divided into cytotoxic humoral antibodies, which have been regularly identified in all virus-induced leukaemias, and host-cell-mediated inhibition, which has been shown to operate in all tumour systems studied.

Variation in the cytotoxic sensitivity to tumour-specific humoral antibodies in different lymphomas induced by the same (Moloney) virus has been shown to depend on the concentration of surface antigenic receptors in the same way as we have described for the H-2 system (Klein, Klein and Haughton, 1966).

SOME CONCLUSIONS CONCERNING IMMUNOSENSITIVITY AND TUMOUR CELL RESPONSE TO IMMUNOSELECTION

Table I is an attempt to summarize schematically the response of tumour cells to different defence mechanisms of the host in the H-2 system and also in tumour specific systems. The table also attempts to indicate the response of different kinds of cells to immunoselection *in vivo*, that is, their behaviour following exposure to the immunological response of the appropriate host.

In general, cells carrying a *high* concentration of the pertinent antigens will be sensitive to humoral antibody and complement *in vitro*. This seems to apply both to transplantation antigens like H-2 and to tumour-specific antigens. When exposed to humoral antibodies *in vivo*, these cells are usually inhibited. The probability of enhancement is low. Cells belonging to this category are mostly normal or malignant elements of the lymphatic and haematopoietic series. One may ask whether suppression rather than enhancement of these cells by humoral antibody is dependent upon some physiological property of these cells and not on antigen concentration. This is not likely for two reasons: some carcinomas and sarcomas belong to this category (Boyse, Old and Stockert, 1962); furthermore, the same leukaemic cells that show inhibition when exposed to high concentrations of a "strong" isoantibody such as H-2 may respond with enhancement if the antiserum:cell ratio becomes very low (Boyse, Old and Stockert, 1962) or if they are exposed to antibodies directed against "weaker" antigenic systems, such as transplantation antigens of the non-H-2 type (Möller, G., 1963).

Whenever the cellular concentration of relevant antigens is relatively low in a given tumour-host system, cytotoxic sensitivity becomes low and the probability of enhancement increases. With very low antigen concentrations the likelihood of enhancement becomes very high and even the responsiveness to sensitized

TABLE I

SCHEMATIC SUMMARY OF TUMOUR CELL RESPONSES TO HOST DEFENCE MECHANISMS

Surface antigen concentration	*Probable examples*		*Sensitivity to humoral antibody and complement in vitro*	*Likelihood of enhancement by humoral antibody in vivo*	*Sensitivity to immune lymphoid cells*	*Demonstrated response to immunoselection*
	Transplantation antigens of the H-2 type	*Tumour specific transplantation antigens (TSTA)*				
High	Lymphocytes, bone-marrow cells, leukaemic cells, some carcinomas and sarcomas	Virus-induced leukaemias	+	Low	+	Antigenic loss in H-2 heterozygotes only: decreased antigen concentration with increased immuno-resistance in all categories
Low	Most carcinomas and sarcomas	Polyoma, SV40 MC-induced sarcomas	− or ±	High	+	Antigenic loss in H-2 heterozygotes only; none (in the case of mouse polyoma TSTA) or decreased antigen concentration with increased immunoresistance in all categories
Very low	Long transplanted non-specific tumours	Tumours induced by films (?), some spontaneous tumours (?)	−	Very high	+ or ± (?)	Little likelihood of further change. No "zero" variants known in H-2

host cells may be somewhat impaired although there is no definitive evidence about this.

Any classification in categories of this type must remain somewhat arbitrary and the situation is dynamic, with no sharp border-lines, and depends on the actual experimental conditions which involve variables other than cellular antigen concentration. Nevertheless, it seems timely to attempt some classification of this type, and antigen concentration seems to be the most relevant inherent variable of any given cell-type-antigen-host system. In the case of the H-2 antigens, there is a good deal of evidence to support the scheme presented. For the tumour-specific transplantation antigens the evidence is more preliminary.

Concerning the response to immunoselection, complete antigenic losses have been unequivocally demonstrated only in cells that are heterozygous for the H-2 complex. In all other systems cell populations subjected to prolonged immunoselection tended to respond by the emergence of cell variants characterized by a decreased antigen concentration but unchanged specificity. The only system where prolonged selection pressure was quite unsuccessful in bringing about any quantitative or qualitative change in antigenicity is that represented by specific antigen(s) of mouse polyoma tumours (Sjögren, 1964). This may indicate that the full tumour-specific transplantation antigen complex represents some cellular constituent essential for the viability and/or the neoplastic properties of the cell of the polyoma tumour in the mouse.

THE POSSIBLE ROLE OF HOST DEFENCE (SURVEILLANCE) MECHANISMS IN ONCOGENESIS

Experiments on the immunological tumour-host relationship in transplantation systems can be considered as models, at best, of the various cell-host relationships that may prevail during the process of oncogenesis, but they give no direct information of what actually

does happen. They may be helpful in suggesting experiments but they do not replace the need for direct experimentation.

In discussing the possible role of immunological responses of the host during oncogenesis we will follow the same pattern as in the previous sections and attempt to consider humoral and cell-mediated reactions separately.

With regard to the role of *humoral* antibodies, some of our recent experiments on the Moloney system may be pertinent (Klein and Klein, 1965; 1966). Inoculation of the Moloney virus into adult mice of various strains induces the formation of antibody in high titres in approximately 30 days. (In our experiments, antibody was measured by cytotoxicity or immunofluorescence, with established Moloney lymphoma cells as targets.) In newborn mice, on the other hand, antibody formation was deficient or delayed and a large proportion of the animals failed to respond at all. When tested by a second inoculation of the virus as adults, the previously non-responsive animals still failed to react and thus appeared to be truly tolerant.

The non-responsive animals also failed to react in transplantation tests against antigenic tumour cells and did not develop virus neutralizing antibodies. Similar findings have been made previously by Rubin with avian lymphomatosis virus (Rubin, 1962). He found that infection through the egg was associated with persistent viraemia, absence of antiviral antibody and a sixfold increase of visceral lymphomatosis. Immunological tolerance seems to prevail in two other vertically transmitted virus systems: AKR mice, transmitting the Gross virus from parent to offspring are unable to react against the cellular antigen specific for this type of leukaemia (Axelrad, 1965), and mouse strains transmitting the mammary tumour agent through the milk cannot be made resistant against the mammary cancers that arise although agent-free sublimes of the same strains can be readily immunized (Attia, DeOme and Weiss, 1965).

We have tried to find out if humoral antibodies play an

inhibitory or enhancing role for the development of leukaemia in mice given the Moloney virus. When mice which had been inoculated with the virus neonatally were compared (Klein and Klein, 1965), it was found that animals with significant antibody levels tended to develop leukaemia later than did tolerant animals (animals with little or no antibody). Both these categories did develop leukaemia, and the different time of onset was restricted to the length of the latency period. This indicates that humoral antibodies are fighting a losing battle against the development of the virus-induced leukaemia. This conclusion was strengthened by other experiments in which 0·05–0·1 ml. mouse anti-Moloney serum was injected twice weekly for two months into mice inoculated with the virus at two weeks of age. There was a clear-cut delay in the appearance of leukaemia, and a reduction in the final incidence. These experiments show that humoral antibodies do not seem to play an enhancing, positive role in this system but they may have a certain inhibitory effect.

It has been postulated that immunological surveillance mechanisms play an important role in eliminating potentially neoplastic, antigenic tumour cells arising by spontaneous cellular changes or as a result of chemical or viral oncogenesis in the adult organism; this was also suggested as the evolutionary basis of the homograft reaction (Burnet, 1964). If this is so one may expect, on the basis of the information available from the work on transplantation systems, that cell-mediated host reactions will play a more important role in surveillance than do the humoral antibodies. This expectation is supported experimentally by the observation that thymectomy of newborn animals may increase the incidence of both chemically induced and virally-induced tumours without necessarily affecting the production of humoral antibodies directed against the oncogenic virus. (Ting and Law, 1965). It is not known, however, if immunological surveillance mechanisms are the only ones that the organism has at its disposal to eliminate potentially neoplastic cells. The efficiency of immuno-

logical surveillance is hampered by several factors: this mechanism can only be effective if the neoplastic transformation has changed the cell surface in such a way as to make it clearly antigenic, that is, foreign to the antibody-forming system. This is more pronounced in some cases than in others, as shown by the wide variation in the "strength" of antigenicity of different tumours (*vide supra*). For example, it is much easier to build up resistance in syngeneic mice to methylcholanthrene-induced sarcomas than to sarcomas induced by the implantation of films, and the degree of resistance obtained is higher in the former than the latter. Lymphomas induced by the Moloney agent are much more "strongly" antigenic than morphologically indistinguishable lymphomas induced by the Gross virus. Even in cases where a tumour is clearly antigenic, immunological surveillance can only be efficient if the specific antigen(s) reach the antibody-forming centres in time. A number of restrictions can be envisaged that limit the efficiency of this afferent arm. The same is true for the efferent arm: the immune response has to act on the target in time and must hit it in the right way, not in the left-handed way as sometimes happens when enhancement instead of rejection is achieved. Enhancement probably occurs in cases in which the humoral antibodies reach the target first and, by coating the foreign antigenic sites, diminish antigen release (afferent inhibition) and, by preventing recognition and/or attachment, protect from host-cell mediated graft destruction (efferent inhibition).

This reasoning leads to the conclusion that immunological surveillance, although it undoubtedly exists and probably plays a more important role in some systems than in others, can hardly be the only mechanism established by evolution to protect from potentially neoplastic cells. Other and more locally efficient mechanisms must be looked for. One may envisage tissue homoeostatic forces, capable of eliminating cells with a changed surface structure and correspondingly impaired ability to respond to the growth-regulating mechanisms of the organism. Immuno-

logical responses may indeed be a special case of a more general mechanism, as mentioned above. The work of Hellström and others on syngeneic preference and allogeneic inhibition (Hellström 1963, 1964; Hellström and Hellström, 1965; Möller, 1965*a*, 1966) may reflect the possible existence of some such mechanisms. The findings of Erna Möller suggest that there may be a connexion between "allogeneic inhibition" and immunological response. The cell-mediated immune responses may represent a specialized function inherent in the immunologically competent cells working within the larger framework of a more general mechanism of tissue homoeostasis.

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THE INFLUENCE OF NEONATAL THYMECTOMY AND THYMUS GRAFTING ON SPONTANEOUS AUTO-IMMUNE DISEASE IN MICE

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Two forms of auto-immune disease have been shown to develop spontaneously in laboratory mice. The NZB strain all show an auto-antibody type of haemolytic anaemia, which develops after 3 months of age and is characterized by a positive antiglobulin test and circulating antibody (Helyer and Howie, 1963*a*; Holmes and Burnet, 1963). Certain groups of F_1 hybrids derived from the NZB strain, notably NZB × NZW hybrids, all develop a disorder after 6 months of age which closely resembles a lupus nephritis and which is characterized by a positive lupus erythematosus cell test (L.E. test) (Helyer and Howie, 1963*b*). The two patterns of development and the subsequent courses they follow are distinctive and individually predictable. The patterns of disease can be readily traced during life by a number of simple laboratory tests that are recognized as acceptable attributes of auto-immune disease These two strains of mice, the NZB and the NZB × NZW hybrids, can be conveniently, if somewhat loosely, described as auto-immune strains.

Interest has developed recently in a possible pathogenetic relationship between the thymus and auto-immune disease. The thymus has been shown to have an important influence on the development of normal immunological activity during the

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neonatal period, and thymectomy in newborn mice induces a profound impairment of immunological function (Miller, 1964). If auto-immune disease arises from a derangement of normal immune mechanisms it would be of interest to know whether or not auto-immune phenomena, as well as normal immunological activity, are affected by neonatal thymectomy.

It has been suggested (Burnet and Holmes, 1964) that a morphological expression of the thymic abnormality in auto-immune disease is the development of germinal follicles in the thymic medulla of NZB mice. These follicles appear at about the time the auto-immune disorder commences. Similar observations in certain human disorders, possibly of auto-immune origin, have been made (Editorial, 1965) and these observations support the contention that there may be a causal relationship between the medullary hyperplasia of the thymus and the auto-immune disease.

This paper describes some observations on the effects of neonatal thymectomy and neonatal exchange grafting of thymus glands in normal and auto-immune groups of mice. Some preliminary results of this study have already been published (Helyer and Howie, 1963*c*).

MATERIALS AND METHODS

CBA/T6 mice (Carter, Lyon and Phillips, 1955) were used as normal animals. It was shown, using the auto-immune markers described in this paper, that in this strain there is no evidence of auto-immune disease throughout life or at post mortem. Both the NZB strain and NZB × NZW hybrids were used as auto-immune animals.

The technique of thymectomy and thymus grafting used has been described (Helyer and Howie, 1963*c*). Groups of mice of both sexes were prepared according to the categories in Table I.

TABLE I

CATEGORIES OF ANIMALS PREPARED IN THYMECTOMY AND THYMUS-GRAFTING EXPERIMENTS

Procedure	*Source of neonatal thymus*	*Neonatally thymectomized animals*
Thymectomy		CBA/T6 NZCW O.U.† outbred stock
Thymus grafts: Normal thymus	CBA/T6 CBA/T6 CBA/T6	CBA/T6 NZB NZB × NZW
Thymus grafts: Auto-immune thymus	NZB NZB × NZW NZB NZB × NZW	NZB NZB × NZW CBA/T6 CBA/T6

†O.U. = Otago University.

Where the graft was of the same strain as the recipient, animals were treated both with an isograft and also by replacement of the ablated thymus into the same animal from which it was removed. This latter procedure is described as thymic reinstatement.

The mice were weighed twice weekly, or more frequently if the animal was ill. The animals were examined by a special screening procedure at least every two months. This comprised direct antiglobulin tests, direct L.E. tests, latex nucleoprotein tests and haematocrit estimations. The techniques used in these tests have been described elsewhere (Helyer and Howie, 1963*c*). The mice were maintained to their full span of life and were only killed because of progressive loss of weight or obvious ill health. At post mortem, in addition to the full screening procedure, a blood urea was estimated and the urine was examined for protein. Post-mortem examination was supplemented by full histological study using sections cut at 2·0 μ and stained with haematoxylin and eosin, Masson's trichrome, alcian blue, periodic acid-Schiff (PAS), and Tripas technique.

RESULTS

NEONATAL THYMECTOMY IN NORMAL MICE

Male and female CBA/T6 mice were thymectomized within 24 hours of birth and studied throughout life. There is a 65 per cent mortality before weaning in this strain. The survival pattern following weaning is the same for both sexes, and combined curves for thymectomized and non-thymectomized, male and female CBA/T6 mice are shown in Fig. 1: in the thymectom-

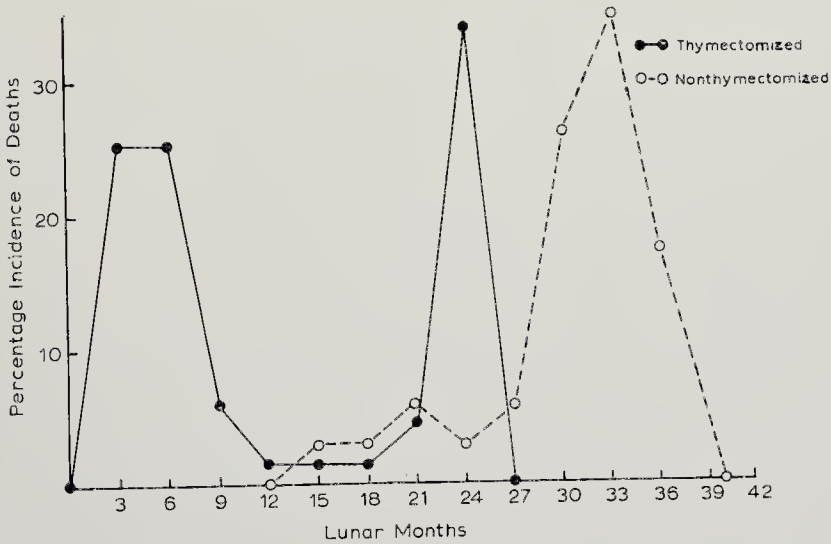

FIG. 1. Percentage distribution of age at death in neonatally thymectomized CBA/T6 mice.

ized group the distribution is bimodal. About 50 per cent of the thymectomized mice have a short survival time and die between the third and fifth months. These animals progress reasonably normally until they deteriorate suddenly over a few days, and develop a flaccid paraplegia 24–48 hours before death. At post mortem these animals show granulomatous necrotic lesions in the liver, spleen, lymphoid tissue, bone marrow and meninges. These lesions are identical with those described by East *et al.*,

(1963) as closely resembling what is found with mouse hepatotrophic virus-1 (MHV-1). Focal necrotic lesions can be demonstrated in the white matter of the spinal cord and the cerebrum. In both males and females, weak positive Coombs' tests and positive latex nucleoprotein tests are seen terminally in some of the animals with liver necrosis and paraplegia dying between the second and fifth months. Occasionally anaemia develops terminally in this group. Unequivocally positive L.E. tests are not seen, but one doubtful L.E. cell was seen on one occasion in each of two animals.

The mean survival time of thymectomized animals living beyond six months is a little shorter than that of non-thymectomized mice of the same strain. No positive auto-immune markers are seen throughout their lives, except for a few animals that develop a positive latex nucleoprotein test terminally. Post mortem findings are the same as in the non-thymectomized group.

With NZCW mice and an outbred stock strain, approximately 70 per cent of males and females survived thymectomy for twelve months or more. The remainder died between two and six months of age with liver necrosis, and wasting disease associated with diarrhoea. The morbid anatomical changes and the incidence of positive auto-immune markers resemble those of the CBA/T6 group of mice.

NEONATAL THYMECTOMY IN NZB MICE

This strain normally develops an auto-immune type of haemolytic anaemia, with a positive Coombs' test and circulating auto-antibody, at about $4\frac{1}{2}$ to $5\frac{1}{2}$ months of age. Two to four months later these abnormalities are followed by a progressive anaemia. There is an 80 per cent mortality of neonatally thymectomized mice of this strain before weaning. Seventy per cent of the animals that survive weaning, both males and females, die within

4 months. They are undersized, wasted and many develop diarrhoea. They do not become paraplegic and only a small percentage show liver necrosis of the MHV-1 type. Animals that survive for longer than four months show the typical pathological features of haemolytic disease. Before death, many of the short-lived mice—the majority in this group—develop sustained strongly positive Coombs' tests, circulating auto-antibody and anaemia. These abnormalities appear at least two months earlier than in non-thymectomized animals. Thymectomy appears to induce an earlier onset of disease in this group of short-lived mice but there are insufficient animals surviving for longer than four months to be certain that this applies to all thymectomized NZB mice.

The study of the influence of thymectomy on the auto-immune disorder of this strain of mice is complicated by the widespread incidence of wasting disease. It is apparent, however, that neonatal thymectomy does not prevent the onset of auto-immune haemolytic anaemia in NZB mice, and the evidence presented suggests that, after thymectomy, the time of onset may, in fact, be earlier than in non-thymectomized animals.

NEONATAL THYMECTOMY IN NZB × NZW HYBRID MICE

This hybrid strain develops a condition closely resembling human systemic lupus erythematosus. The animals show positive L.E. tests and Coombs' tests. They develop increasing proteinuria, a rising blood urea and progressive anaemia, and they die with the classical histological features of lupus nephritis.

These mice survive thymectomy remarkably well and have a mortality of 50 per cent before weaning. In both sexes the slopes of the weight curves and the final maximum weights are very similar for non-thymectomized and thymectomized animals (Fig. 2). A very small proportion of animals die within four months with liver necrosis of the MHV-1 type, and these have

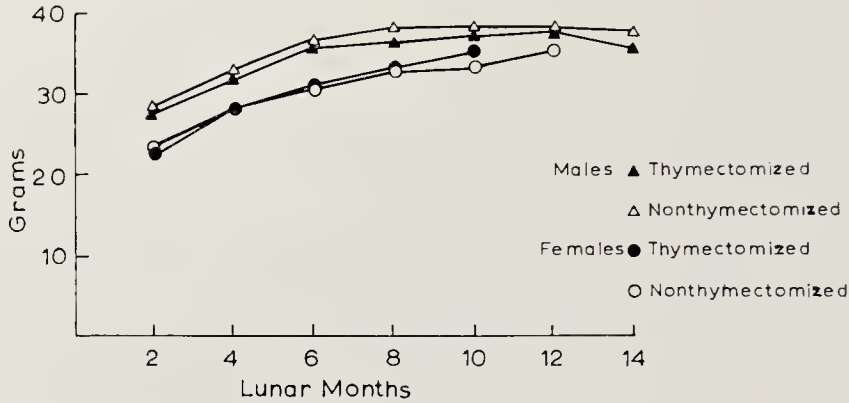

FIG. 2. A comparison of average-weight curves in thymectomized and non-thymectomized NZB × NZW mice.

been excluded in calculating survival data. The average survival time for the thymectomized group of animals is considerably reduced (Table II). In 21 females the average survival time was reduced by 60 days and in 27 males by 72 days.

TABLE II

MEAN SURVIVAL TIMES OF NZB × NZW MICE WITH AND WITHOUT NEONATAL THYMECTOMY, AND WITH NEONATAL THYMUS GRAFTS FOLLOWING THYMECTOMY

	Arithmetical Means of Survival (days)	
	Male (No of animals)	Female (No. of animals)
Untreated	439 (90)	280 (250)
Thymectomy	367 (27)	220 (21)
Thymectomy & Reinstatement of Thymus	454 (6)	273 (7)
Thymectomy & Graft of CBA/T_6 Thymus	427 (25)	286 (24)

The clinical presentation of the disorder, particularly in females, is also modified by thymectomy. In some non-thymectomized animals mild to moderate subcutaneous oedema occurs terminally, but in the thymectomized group severe and widespread oedema,

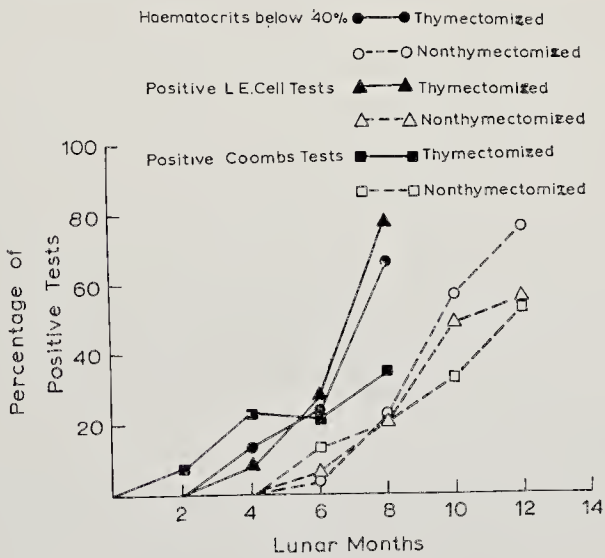

FIG. 3.

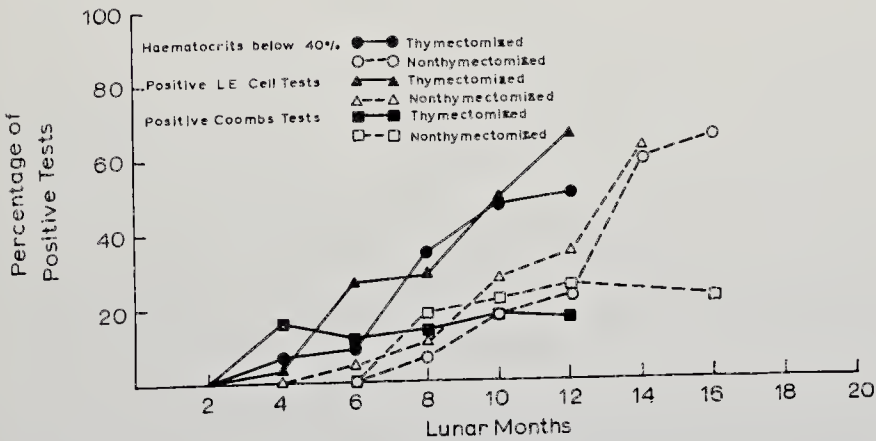

FIG. 4.

FIGS. 3 and 4. Comparison of the percentage incidence of haematocrits below 40 per cent, positive L.E. tests and positive Coombs' tests in thymectomized and non-thymectomized NZB × NZW mice. Fig. 3 = females. Fig. 4 = males.

pleural effusion and ascites are particularly frequent in females, and the clinical appearance is analogous to the appearance of the nephrotic syndrome. The incidence of auto-immune markers and anaemia in thymectomized and non-thymectomized animals is shown in Fig. 3 (females) and Fig. 4 (males). In both sexes, positive auto-immune markers and anaemia appear at least two months earlier in the thymectomized mice. This parallels the 60–70 day reduction in mean survival times of thymectomized animals. The slope of increment of the percentage of positive auto-immune marker tests plotted against time is very similar in the two groups, suggesting that thymectomy has induced an earlier onset of the disorder in all members of the group but has not altered the pattern of development of the disease. Finally, there is evidence from histological examination of the kidney that there is an increase in acuity of the renal lesions in the thymectomized animals.

It is concluded, therefore, that neonatal thymectomy brings about an earlier onset and enhances the acuity of auto-immune disease in the NZB × NZW hybrid mouse.

THYMUS GRAFTS

"NORMAL" THYMUS GRAFTS IN NORMAL ANIMALS

CBA/T6 mice were treated either by grafting according to the procedure described as thymic reinstatement (this volume, p. 362), or with an isograft. With both these procedures the animals showed a normal survival pattern analogous to that seen in the non-thymectomized mice. They did not develop paraplegia or hepatic necrosis and showed no positive auto-immune markers.

NORMAL OR AUTO-IMMUNE GRAFTS IN AUTO-IMMUNE ANIMALS

NZB mice and NZB × NZW hybrids were treated by thymic reinstatement, or by grafting of thymus glands from the same strain or from CBA/T6 mice. The pattern of auto-immune

disease developing in these grafted animals is restored to the pattern in non-thymectomized animals, irrespective of whether the thymic graft is normal or auto-immune. Thymus grafting eliminates wasting disease in NZB mice and in NZB × NZW hybrids it restores the survival time to normal (see Table II). The grafted normal thymus glands do not in any way affect the development of auto-immune markers, or modify the course of the disease in the auto-immune groups of mice.

AUTO-IMMUNE GRAFTS IN NORMAL ANIMALS

CBA/T6 mice, thymectomized within 24 hours of birth, were grafted with thymus glands from newborn NZB, and NZB × NZW hybrid mice. The survival pattern of these grafted mice

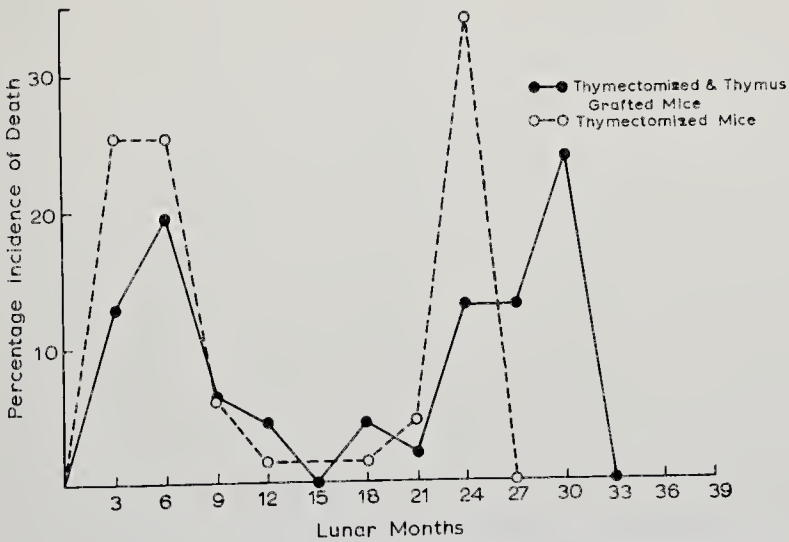

FIG. 5. Percentage distribution of age at death in neonatally thymectomized CBA/T6 mice with and without neonatally grafted NZB or NZB × NZW thymus glands.

shows a bimodal distribution with approximately equal numbers of animals with short and long survival times (Fig. 5). A higher proportion of females are in the short-survival group, and there is no apparent difference between the groups grafted with NZB or

NZB × NZW thymus glands. The bimodal distribution of survival closely resembles that seen with CBA/T6 thymectomized mice but, in contrast, in the short-survival group of grafted animals no clinical paraplegia precedes death. Weight curves in the short-survival group show a slower rate of weight gain than in the long-survival group, and a very brief period, if any, of weight loss before death. The long-survival group shows the same rate of weight gain as non-thymectomized animals and eventually the long-survival group reaches and even exceeds the average maximum weight of the non-thymectomized mice.

TABLE III

INCIDENCE OF POSITIVE AUTO-IMMUNE MARKERS IN NEONATALLY THYMECTOMIZED CBA/T6 MICE WITH GRAFTED NEONATAL NZB OR NZB × NZW THYMUS GLANDS

Positive Coombs' Test	78 %
Positive L.E.Cell Test	66 %
Positive Latex Nucleoprotein Test	66 %
3 of 3 Markers Positve	33 %
2 of 3 Markers Positive	38 %
1 of 3 Markers Positive	26 %
0 of 3 Markers Positive	3 %

In contrast to thymectomized CBA/T6 mice, the mice grafted with auto-immune thymus glands show strongly positive and persistent auto-immune markers. The incidence of single positive markers and combinations of positive markers is shown in Table III. It is most significant that two-thirds of the animals have positive L.E. tests. The patterns of behaviour of the markers are very variable in different animals and at different times during the life-span of individual animals. Some examples of this are shown in Table IV. In the short-survival group a strongly positive

TABLE IV

VARIETY OF PATTERNS OF LABORATORY TESTS IN THE LIFE-SPAN OF CBA/T6 MICE GRAFTED NEONATALLY WITH NZB OR NZB × NZW THYMUS GLANDS

Months of age	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Male CBA/T_6 With NZB×NZW Thymus																														
Coombs Test			—	++																										
Haematocrit %			51	36																										
L.E. Cell Test			+	+																										
Latex N.P. Test			++++	++++																										
Female CBA/T_6 With NZB×NZW Thymus																														
Coombs Test		++++		++++		++++		++		++		++																		
Haematocrit %		33		29		37		42		44		47																		
L.E. Cell Tests		+		+		+		+		+		+																		
Latex N.P. Test		+		++		++		+		—		++++																		
Male CBA/T_6 with NZB Thymus																														
Coombs Test				++++		++++		++++	++++		++++			++++		++++		++++		++			++		++++	++			++	++
Haematocrit %				36		37		34	30		34			40		39		40		38			38		36	38			34	32
L.E. Cell Test				+		—		—	—		—			—		—		—		—			—		—	—			—	—
Latex N.P. Test				—		—		—	—		—			—		—		—		—			—		—	—			—	—
Female CBA/T_6 With NZB Thymus																														
Coombs Test				++		±		—	—		±			—		+		±		—			—		—		—		—	—
Haematocrit %				50		43		49	47		46			44		44		44		46			41		43		45		40	43
L.E. Cell Test				—		—		+	—		—			—		+		+		+			+		+		+		+	+
Latex N.P. Test				—		—		+	—		—			++		++++		++		++++			++		++		++++		++++	++

Coombs' test develops within two months, and may be succeeded by positive L.E. tests and strongly positive latex nucleoprotein tests. These are accompanied by an anaemia which, immediately before death, may reach quite profound levels. Animals in this group may show heavy albuminuria and azotaemia. In some animals which survived for longer than three to four months the

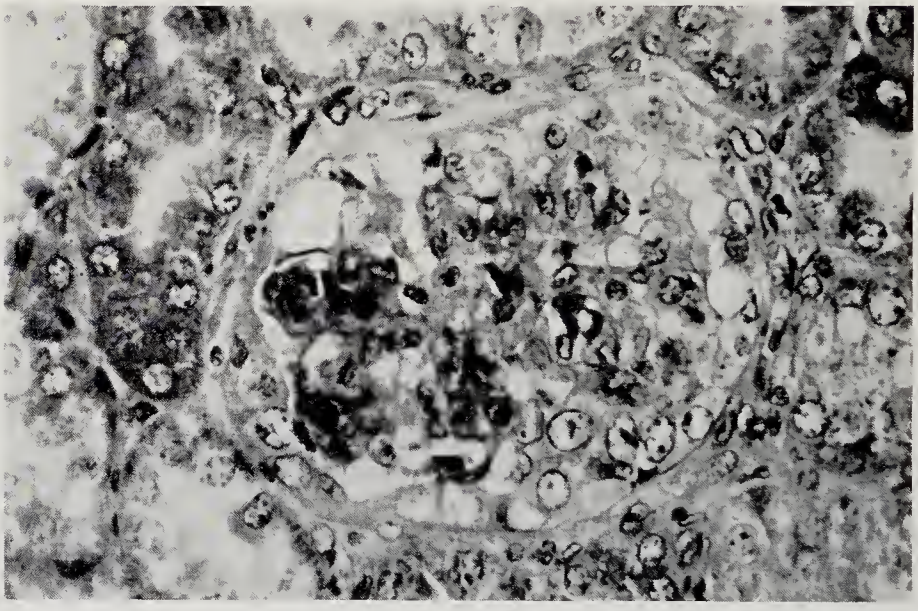

FIG. 6. Glomerulus of male CBA/T6 mouse, exchange-grafted neonatally with an NZB × NZW thymus, and dying at 3 months. Note enlargement of glomerulus with hypercellularity, areas of fibrinoid necrosis at one pole and capsular proliferation with crescent formation. Masson's trichrome. (× 465).

positive Coombs' or positive L.E. tests may persist, either singly or in combination, for two years or more, or may gradually diminish in strength and disappear. In these animals with a prolonged survival time, haematocrit levels may be low in the first few months and then gradually return to normal only to decrease again in the last few months of life, often with associated evidence of renal impairment such as proteinuria and azotaemia.

No histological evidence of granulomatous liver necrosis is present in the short-survival group. In some animals dying with profound anaemia, an entirely different form of liver necrosis, with a centrilobular distribution, is present. This closely resembles the necrosis seen terminally in untreated NZB mice dying with active auto-immune haemolytic anaemia. In the grafted animals

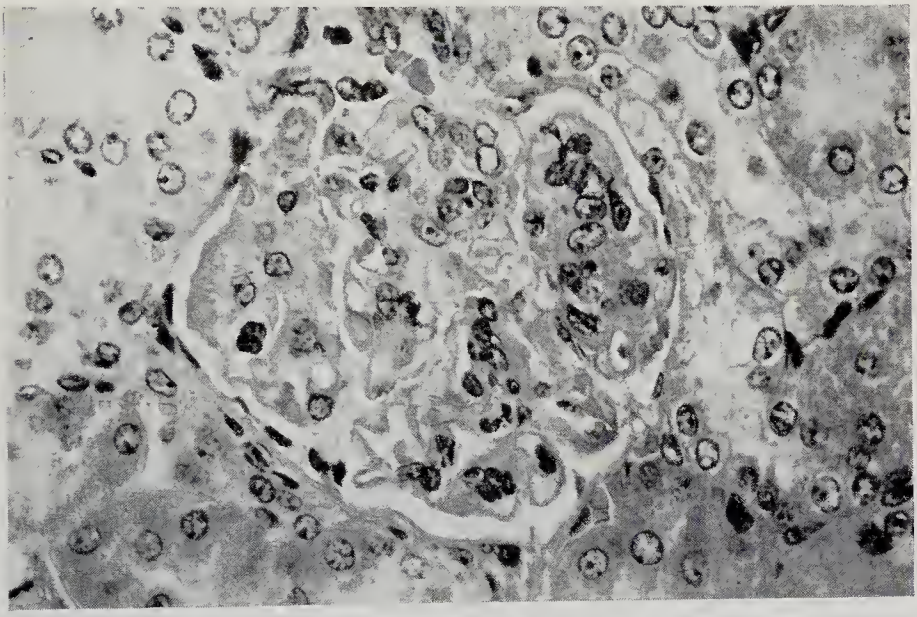

FIG. 7. Glomerulus of female CBA/T6 mouse, exchange-grafted neonatally with an NZB × NZW thymus, and dying at 27 months. Note patchy hypercellularity, focal areas of basement membrane thickening and fibrinoid necrosis. Haematoxylin and eosin. (× 465).

this characteristic type of liver necrosis is linked with a strongly positive Coombs' test and severe anaemia, and is invariably associated with considerable haemosiderin deposition in liver and spleen.

The kidneys show varying degrees of endothelial proliferation in the glomerular tufts and, in some, acute glomerular lesions characteristic of lupus glomerulonephritis are present (Figs. 6 and 7). These include areas of focal fibrinoid necrosis of the basement

membrane and even of segments of tufts together with capsular adhesions and epithelial-crescent formation. Renal and extra-renal vascular lesions are also seen. In older animals the kidneys show varying degrees and stages of development of lupus glomerulitis and glomerulonephritis (Figs. 8, 9 and 10). Lesions at quite widely different stages of development are present in the

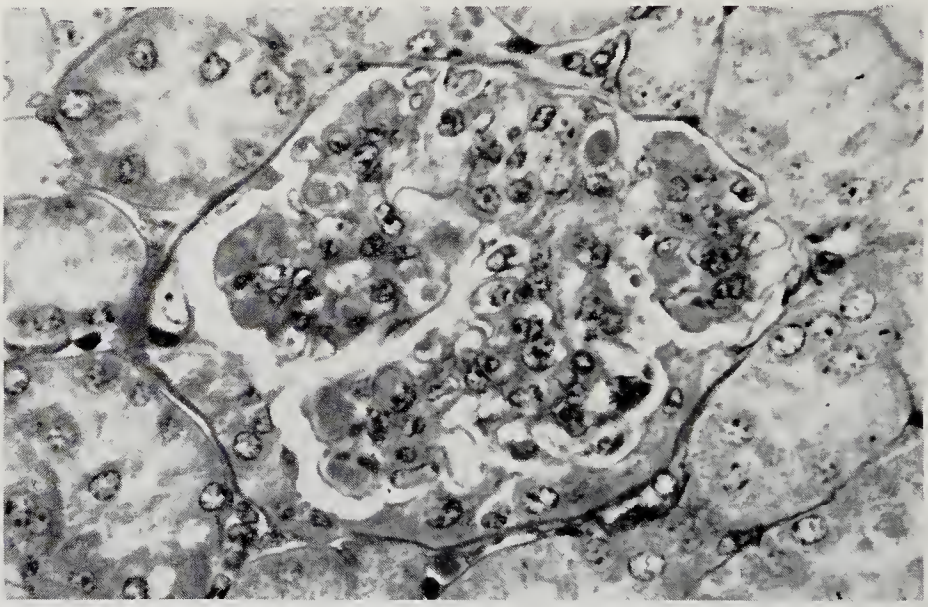

FIG. 8. Glomerulus of male CBA/T6 mouse exchange-grafted neonatally with an NZB thymus and dying at 24 months. Note hypercellularity with basement membrane thickening and hyaline "thrombi". Masson's trichrome. (× 465).

same kidney. Even in a single glomerulus both collagenous sclerosis and obliteration may be present, along with active endothelial proliferation. These findings suggest a long-continued process of episodic character with periods of activity and remission. The episodic nature of the process is also indicated by the fluctuations in incidence of positive markers and low haematocrits, and the divergence in the weight curves of individual animals. With increasing age animals show a greater incidence of

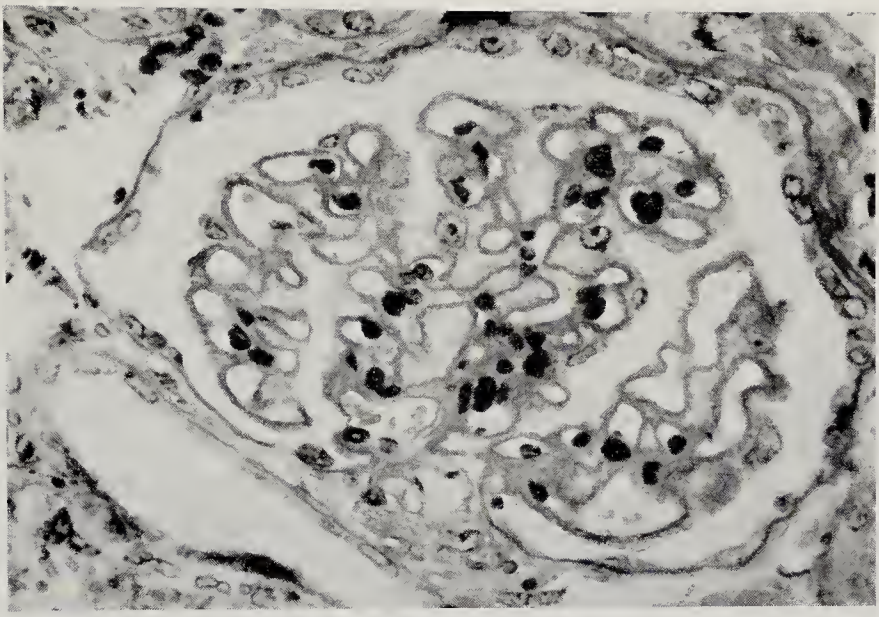

FIG. 9 . Glomerulus of male CBA/T6 mouse, exchange-grafted neonatally with an NZB × NZW thymus, and dying at 23 months. Note uniform type of thickening of basement membrane with fibrinoid necrosis on the epithelial aspect of the membrane. Tripas. (× 465).

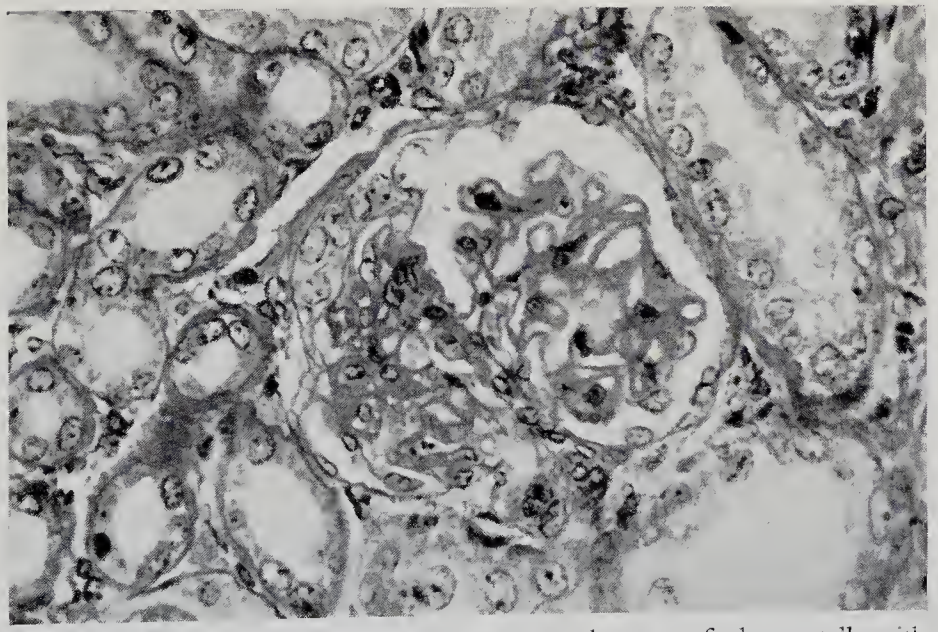

FIG. 10. Glomerulus of female CBA/T6 mouse, exchange-grafted neonatally with an NZB × NZW thymus and dying at 7 months. Note that half glomerulus has undergone collagenous sclerosis and obliteration, the other half shows advanced wire-loop type of thickening of basement membrane. Masson's trichrome. (× 465).

sclerotic glomerular lesions, involving either segments of glomerular tufts or whole tufts.

All the animals studied were killed because of ill health, loss of weight or impending death so that varying degrees of acute thymic involution could be expected to influence the weight of the thymus in animals with both short- and long-survival times. The post mortem weights of the thymic grafts vary from 62 mg. to 5 mg. In some young animals only thymic medulla is present whereas in others, particularly in older animals, a good cortex of thymocytes is seen. All show abundant epithelial-reticular cells, and thymic corpuscles are present. Considerable proliferative activity of medullary cells is seen but no clearly identifiable germinal centres could be identified.

In CBA/T6 mice, grafted neonatally with auto-immune thymus glands, survival in about half the cases is brief and in the other half prolonged. An acute process with strongly positive auto-immune markers may develop early in the animal's life and death may supervene rapidly with severe anaemia or acute lupus nephritis. Alternatively, the animal may flourish and gain weight normally. However, by three to four months, positive auto-immune markers appear which may be transient or may persist until death. Finally, after a variable period of survival, the animal dies with histological evidence of chronic lupus nephritis. In both the short-survival and long-survival group the pattern of development of the disorder in terms of clinical, laboratory and morbid anatomical features is basically the same in both males and females, and uninfluenced by whether the grafted auto-immune thymus is taken from an NZB or NZB × NZW hybrid strain of mouse.

DISCUSSION

In thymectomized CBA/T6 mice a syndrome of paraplegia and granulomatous liver necrosis acts as a clear-cut clinical and pathological marker. Hepatic necrosis has been described in other

normal strains (Parrott and East, 1964; Osoba and Miller, 1964), and also occurs infrequently in the NZB strain and NZB × NZW hybrids, but paraplegia appears to be unique to the CBA/T6 strain at Dunedin. Considerable variation in the incidence and time of onset of the thymectomy syndrome has been reported (Miller, 1964; Parrott and East, 1964), and significant numbers of thymectomized mice escape the disorder. In this investigation 50 per cent of CBA/T6 mice have survival times approximately the same as those of intact animals. These observations could be explained in terms of incomplete removal of the gland, but it is more likely that some selective mechanism, such as varying degrees of immunological maturity at birth, may be an important factor in determining variations in post-thymectomy survival, both within a strain and between strains.

Positive auto-immune markers do not appear in thymectomized normal animals with reinstated or grafted normal thymus glands, but there is a definite incidence of transient weakly positive tests in the short-survival group of thymectomized animals. The presence of these positive tests suggests that the thymus, during the early postnatal months of life, may exert a regulatory influence on self-recognition and suppression of the forbidden clones concerned with auto-antibody production.

In auto-immune animals neither neonatal thymectomy nor the neonatal replacement of the thymus by a normal gland is able to prevent the development of auto-immune disease. With a normal exchange-grafted thymus the disorder develops in the same manner as in intact animals and is not influenced by the graft being normal or auto-immune. With thymectomy the auto-immune process develops both precociously and more acutely. If auto-immune processes are initiated by the development of forbidden clones of immunologically competent cells, either within or under the influence of the thymus, then to accord with these observations such clones must be produced and undergo extra-thymic dissemination during intrauterine life. During the

postnatal period these clones must continue to grow and proliferate independently and to be influenced by the thymus only in a regulatory or inhibitory manner.

In normal animals with grafted auto-immune thymus glands, the development of strongly positive auto-immune markers and of renal changes characteristic of lupus nephritis strongly suggests that the auto-immune phenomena have been transmitted by the graft. If this is interpreted on an immunological basis, then the grafted thymus could have exerted its auto-immune influence in a number of ways: a clone or clones of cells capable of auto-antibody production may have been established from the graft; or the grafted thymus, because of incompetence or dysfunction, or because of a positive humoral influence, may have induced the formation of forbidden clones from the host's population of immunologically competent cells

Alternatively, the evidence might be considered as support for the theory that a transmissible agent, such as a virus, is harboured in the auto-immune thymus. The bimodal survival distribution of the normal animals with auto-immune thymus grafts closely resembles the survival distribution curve of thymectomized normal mice. The MHV-1 type of liver necrosis in the short-survival group of thymectomized normal mice might be due to an unmasking or potentiation of the virus by thymectomy, as suggested by East and co-workers (1963). By analogy, the strongly positive auto-immune phenomena of the short-survival group of grafted animals could result from potentiation of a viral agent by inadequate thymic function. In this group the viral agent would then be responsible for an acute and rapidly fatal illness. In the remainder of the animals, with less impairment of thymic function, a lower grade of auto-immune disorder would develop.

Neither thymectomy nor a neonatally grafted normal thymus prevents the expression of the auto-immune disorder in affected strains of mice. This observation accords with the idea that there is prenatal transmission of a virus. Furthermore, if the absence or

inadequacy of thymic function is capable of potentiating viral activity, then the enhancement of the auto-immune disease by thymectomy is readily explained.

Such a viral hypothesis places a rather different emphasis on the role of the thymus in auto-immune disease. The thymus may provide, at least initially, a residence for the virus and thus the gland could exert a modulating influence on the acuity or chronicity of the pathological process induced. The thymus may subsequently develop histopathological changes which represent part of the general lymphoreticular response to the viral infection. However, on this hypothesis, the thymus itself could not be regarded as the primary site of auto-immune processes.

Finally, it must be concluded that these experimental observations on the role of the thymus in auto-immune disorders in mice are inconclusive. A viral hypothesis is as far from proven, by the evidence provided, as is a hypothesis based on the primary development of forbidden clones.

SUMMARY

(1) A variable proportion of neonatally thymectomized mice die at an early age from a syndrome of wasting, diarrhoea and granulomatous liver necrosis.

(2) In normal mice this thymectomy syndrome is associated with the occurrence of weak positive auto-immune markers, such as positive antiglobulin and antinuclear tests.

(3) In auto-immune strains of mice thymectomy causes the precocious development, and an increase in acuity, of the disorder. This is prevented by a neonatal graft of normal or auto-immune thymus.

(4) Neonatally thymectomized normal mice grafted with newborn auto-immune thymus develop serological and histological evidence of auto-immune disease.

(5) The significance of these findings and their impact on the role of the thymus in auto-immune disease have been discussed in terms of immunological and viral theories.

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THYMIC CHANGES IN NZB MICE AND HYBRIDS

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Since 1962, when we first recognized that abnormal proliferative areas were present in the thymus in NZB mice (Burnet and Holmes, 1962) and appreciated the similarity of these areas to the germinal centres in the thymus in cases of myasthenia gravis in man, we have carried out a number of largely unsuccessful experiments in an attempt to explain the role of the thymus in the disease which occurs spontaneously in these mice.

NZB mice are apparently normal in early life but antibodies can first be detected on their red cells at about 100 days and all the mice have a positive Coombs' test before death. Red cell antibodies giving a positive indirect Coombs' test or a positive-papain-cell agglutination test can be detected in the serum. The antibody-coated red cells have an abnormally short life and are removed from the circulation, primarily by the spleen, after a half-life of one to three days compared with the usual half-life of ten days for red cells in young NZB mice and in normal strains (Long, Pye and Holmes, 1966). The haemolytic anaemia occurring in NZB mice is similar in all respects to the warm-antibody type of haemolytic anaemia occurring in man (Dacie, 1962). Burnet had suggested that the human and murine conditions represent the activity of what he calls "forbidden clones" of cells which are capable of producing antibody against body constituents, in this instance the circulating red cells (Burnet, 1962). The possibility that the emergence of these pathogenic

cells into the circulation is a result of some failure of thymic function has also been suggested.

The occurrence of lesions in the thymus in NZB mice has been well documented (Burnet and Holmes, 1964). The thymic lesions consist of areas of expanded medulla, often with undoubted germinal centres containing mitoses and pyknotic cells and surrounded by a segment of a cuff of small lymphocytes, with a cleared area of medulla outside this. Germinal centres occur singly or in massive lesions where germinal centres of different ages can be found together. These centres, although not as well defined as the germinal centres in man, are undoubtedly similar to those occurring in the spleen or lymph nodes in mice, and have an obvious resemblance to the germinal centres in myasthenia gravis in man.

At the time these observations were made the profound importance of the role of the thymus in immunological capacity was just being recognized (Miller, 1961).

It was also well known at this time that the first abnormalities in leukaemia in AKR mice occurred in the thymus, and that thymectomy carried out before the onset of thymic changes would prevent the subsequent development of leukaemia in these mice (McEndy, Boon and Furth, 1944). It had been shown (Law, 1952; Miller, 1960) that the implantation of a thymus graft in a thymectomized animal restored its susceptibility to leukaemia, and that transfer of a thymus from an AKR parent to an F_1 host increased this susceptibility.

Since the recognition of the occurrence of lesions in the thymus in NZB mice, we have attempted to prevent the disease in the NZB mice themselves and to accelerate the onset in F_1 hybrids of the NZB mouse and other normal strains, but with little success. The thymus lesions are first recognized histologically at about the time the Coombs' test converts to positive, that is, at about 150 days (Burnet and Holmes, 1964). Male mice are often Coombs' positive when no lesions can be found in sections from

the thymus but the females generally show thymic lesions before they become Coombs' positive. We recognize that examination of a small number of sections (generally three) from a thymus and performance of a Coombs' test can give only an approximate indication of the onset of the disorder.

EXPERIMENTS

Our first hope was that we would be able to prevent the development of haemolytic anaemia, recognized by the occurrence of a positive Coombs' test, by thymectomy before any abnormalities were detectable in the NZB mice. As the typical

TABLE I

COOMBS' CONVERSION IN NZB MICE

	Number of mice tested	*Percentage alive, direct Coombs' positive at 255 days*	*50 per cent direct Coombs' positive at day*
Controls	54 (m)	1·9	171
	52 (f)	7·7	189
	21 (m, f)	4·8	199
Thymectomy at 4 weeks	34 (m, f)	48·5	253
Thymectomy at <1 week	26 (m, f)	41·7	256

m = male, f = female.

lesions were not found in the thymus before 100–150 days we first attempted thymectomy in four-week-old mice. Thymectomy is known to be successful in preventing leukaemia in AKR mice. This procedure certainly delayed the time of onset of a positive Coombs' test in our mice, but they all eventually became Coombs' positive. The delay in Coombs' conversion is shown in Table 1.

Our next attempt was to remove the thymus before the mice were four weeks old to eliminate the probable early seeding out of cells from abnormal clones. To avoid complete immunological crippling of the mice, thymectomies were carried out at one to

five days. Thymectomy at this age still had a marked effect on survival with, particularly in females, a considerable number of deaths between 100 and 300 days. The mice that died early were almost all Coombs' negative although we would have expected positive tests in many of them by this age. The mice surviving long enough all eventually became Coombs' positive. It seemed probable that it was necessary to cripple the mouse immunologically before we could prevent the development of the autoimmune disease (Holmes and Burnet, 1964*a*).

We also tried to enhance the disease in NZB mice by implanting additional thymus tissue. Grafts of three to six additional thymuses were implanted subcutaneously into four-week-old and one-day-old NZB mice and the Coombs' reaction was measured serially in these animals. In our first experiment, with a group of 20 mice, Coombs' conversion from negative to positive occurred rather early, and florid lesions were found in most of the grafted thymuses. In subsequent experiments Coombs' conversion occurred at the normal age but, again, florid lesions occurred in the thymus grafts.

Because of the early time of onset of the disease in the NZB mouse we thought that it would be worth trying to modify the time of onset in F_1 hybrids, and we have usually used the (NZB × C3H)F_1 hybrid in this work. In these hybrids a positive Coombs' test never occurs before the age of 10 months and very seldom before 12 months; most females first show a positive Coombs' test between 12 and 18 months and some males between 18 months and death. Almost all surviving females become Coombs' positive at some time although lymphatic leukaemia causes a reversion of the Coombs' test in many of them; only a small number of the males survive long enough to become Coombs' positive (Holmes and Burnet, 1964*b*).

More recently we have used (NZB × T6)F_1 hybrids to carry out chromosome analyses. Coombs' conversion in these hybrids and in the (NZB × C3H)F_1 hybrids has a similar pattern.

Subcutaneous neonatal grafts grow well in the (NZB × T6) hybrids and, as in the NZB parent, the graft grows to a larger size in females. We have not carried out chromosome analyses in intact hybrids but have assumed from the work of others, for example Wakonig-Vaartaja and Metcalf (1963), that the lymphoid element in the graft is replaced in a short time by host F_1 lymphoid cells.

Grafting of neonatal NZB thymus tissue to intact, four-week-old F_1 hybrids has not accelerated the onset of haemolytic anaemia (Table II). Although the reticular and epithelial element of the graft still presumably consists of NZB cells these F_1 mice

TABLE II

COOMBS' CONVERSION IN (NZB × C3H)F_1 AND (NZB × T6)F_1 HYBRIDS

	Number of mice tested	*Per cent direct Coombs' positive by 365 days*
Control		
NZB × C3H	60	11·7
NZB × T6	53	5·6
Thymus graft or spleen + thymus graft	65	6·2
Thymectomy + thymus graft	20	5·0
X-irradiation + thymus graft	34	> 41·2

did not become Coombs' positive early, and when they were killed at twelve months they were still fat, healthy and with spleens of normal weight. The natural host thymus and the graft thymus were examined histologically and both usually showed abnormalities. The standard F_1 type of lesion is recognizably different from most of the lesions seen in NZB thymuses, and the lesions in the NZB thymus grafts implanted into F_1 hosts were usually of the F_1 type.

A variety of modifications, including implantation of NZB grafts neonatally, multiple grafts (three to six thymuses) at birth or at four weeks and grafts under the kidney capsule, have been

tested. None of these modifications has significantly accelerated conversion to the Coombs' positive state.

Following the publication of Helyer and Howie's work (Helyer and Howie, 1963) describing their success in producing positive findings indicative of auto-immune disease in T6 mice thymectomized at birth and grafted immediately with NZB tissue, we attempted some similar experiments. We performed neonatal thymectomy in $(NZB \times C3H)F_1$ and $(C3H \times C57BL)F_1$ hybrids and implanted subcutaneous grafts of NZB thymus either at the time of operation or one month later. These experiments are still in progress but we have found no positive Coombs' tests before 12 months and no kidney disease so far. In a small number of the mice already examined histologically, thymectomy has been complete and the grafts have grown successfully.

In experiments in collaboration with Miss Lorraine Sibthorpe, we have investigated the transfer of the Coombs' positive condition to young NZB animals by transfering to them syngeneic spleen cells from old Coombs' positive donors. These experiments have shown a consistent conversion to positive of the Coombs' test in the young animal two to four weeks after transfer, provided that a large enough dose of spleen cells is given (approximately 20×10^6 cells). Similar results have been obtained when F_1 recipients are given cells from spleens of old NZB or old F_1, Coombs' positive donors. Suspensions of cells from lymph nodes, thymus, bone marrow or buffy coat have never given positive tests in the recipients.

In the syngeneic combinations, the young animals, all with positive Coombs' tests after transfer of spleen cells from Coombs' positive donors, were killed four to six weeks after the transfer. They showed a considerable increase in spleen weight which histologically corresponded to an increase in the red pulp of the spleen. The thymus usually showed no recognizable lesions—the mice were all killed at least two months before spontaneous lesions would be expected. In one series, however, three recipient

mice showed undoubted thymic lesions which consisted of cleared circular regions and, in one of the mice, these regions contained two germinal centres. In several other series no thymic lesions were present. In another series the recipients showed Till-McCulloch-like plaques of reticulum-cell tumour in the spleen. Coombs' conversion occurred normally in these mice but, again, there were no lesions in the thymus or anywhere else in the body.

TABLE III

COOMBS' CONVERSION IN IRRADIATED (NZB × C3H)F_1 AND (NZB × T6)F_1 HYBRIDS

	Number of mice irradiated	*25-day survival (per cent)*	*Percentage of survivors with direct Coombs' test positive*	*Coombs' conversion at day:*
No graft	62	45·1	0	—
Foetal liver graft	51	62·7	6·3	97†, 184†
Foetal liver + thymus graft	41	82·7	41·2	86, 86, 107†, 107, 108, 137, 137, 141, 155, 159, 165†, 204, 324

† Single positive or intermittent positive tests.

In our most recent attempts to produce an early onset of the disease in F_1 hybrids we have damaged the immunological tissue of the F_1 host with X-irradiation before implantation of the NZB parental tissue. These experiments have been carried out in collaboration with Dr. Erwin Diener and Miss Lorraine Sibthorpe. The work is still in progress and many more controls are needed to interpret the results but, because we are now succeeding in obtaining early Coombs' conversion in F_1 hybrids, we are presenting a preliminary report on these experiments. The results obtained so far are shown in Table III.

Following X-irradiation with an approximately median lethal dose (LD_{50}), (NZB × C3H)F_1 and (NZB × T6)F_1 hybrids were

divided into three groups. One group served as a control, one was restored with foetal liver from NZB donors and the third was given NZB neonatal thymus grafts in addition to foetal liver. This dose of X-rays did not cripple the surviving mice immunologically, as the controls showed normal haematocrit levels and circulating lymphocytes, and a normal primary response to flagellar antigen when tested several weeks after irradiation.

More than a third of the mice receiving thymus grafts have already converted to the Coombs' positive state and two doubtful positive tests have been recorded in mice receiving only NZB foetal liver. These positive tests are occurring extremely early compared with the normal conversion time in NZB mice. Dr. Diener has found that both the Coombs' positive and the Coombs' negative recipients of thymus grafts and foetal liver are chimaeras with both donor and recipient cells in the bone marrow, the thymus and the thymus graft. Further combinations of different types of donor and recipient erythropoietic and lymphoid-tissue grafts should provide interesting results.

DISCUSSION

The discovery that thymic lesions occur regularly in NZB mice and their hybrids has not enabled us definitely to implicate the thymus in the process by which auto-immune manifestations develop in these mice.

There are some definite negative results. Early thymectomy, proved complete by histological examination, still allows the development of the Coombs' positive state in these mice. Transfer of spleen cells from a male NZB mouse with a direct positive Coombs' test can give regular conversion of the Coombs' test to positive in young syngeneic recipients *without* producing visible lesions in the thymus. Many hybrids show typical, and in the case of NZB × NZW hybrids gross, thymic lesions without the development of the Coombs' positive state.

The only unequivocally positive results we have obtained that can be ascribed to thymic influence are in irradiated animals and are very difficult to interpret until further combinations have been tested. These results suggest that, in the irradiated animal, cells capable of pathogenic activity and the production of Coombs' antibody are released by the implanted infant NZB thymus. This is far from proven but we are greatly impressed by the evidence of exceptional epithelial activity in some of the grafts. A variety of experiments are in hand, the tools needed to interpret the results—karyotypic analysis and the allotypic examination of the eluted Coombs' antibody—are available and we hope that the continuation of this work will contribute something significant to the problem of the relative importance of lymphopoiesis and the production of epithelial hormone in the thymus.

In our work with multiple thymus grafts we have found, almost regularly, gross pathological changes in the grafts. These changes are reasonably uniform in all grafts, and more active than the lesions that occur in the normal thymuses. This finding strongly suggests (a) that the grafts are more susceptible to the process producing the lesion than is the anatomically normal thymus and (b) that the grafts allow an increased movement through the circulation of whatever is responsible for production of the lesion.

The thymus may only have a remote background role to play in auto-immune disease and the lesions we have been interested in may be only secondary markers of the presence of an auto-immune process in the body. We hope, however, that the five or six years' work on the NZB mice and their hybrids has been enough to show what a valuable model they are for studying auto-immune disease in man. Irrespective of the role of the thymus in the auto-immune process, we can confidently predict that these strains of mice will allow a progressively more complete analysis of this process.

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DISCUSSION

Woodruff: There is another marker of auto-immune disease which Dr. Holmes has been using in Melbourne, and Dr. E. Lindsey and I have been using in Edinburgh for about a year. This is the red cell survival time. By using two labels—^{51}Cr and ^{32}P—it is possible to follow two populations of red cells, those of the host animal itself and those injected into it.

Dr. Lindsey and I have been doing some other experiments which are, in a sense, the converse of those described by Dr. Holmes. Instead of transplanting cells from Coombs' positive to Coombs' negative mice, we have been looking at the effect of irradiating Coombs' positive mice (with a dose of 800 rads) and then repopulating them with a mixture of marrow and spleen cells from mice that are Coombs' negative.

In our first experiment we used syngeneic donors, namely young NZB mice which had not yet become Coombs' positive, and gave the recipients a total dose of either 1×10^8 or 5×10^7 cells. Eleven mice survived the procedure. These were between six and twelve months old at the time the experiment was begun. Eight of them became Coombs' negative. Two of these are still alive and Coombs' negative three months and eight months respectively after the cell infusion, and

four others remained Coombs' negative up to the time of their death three to seven months after the cell infusion.

In our second experiment we followed the same plan except that we used (NZB×T6)F_1 hybrids as our donors. Four of our animals are still alive after about five months and all of these have been converted to a persistently negative Coombs' test. These animals are now between twelve and fourteen months old. I predict that they will remain Coombs' negative until they die of old age. What we really want to show is that these mice are chimaeras—this is why we used the (NZB×T6) hybrid—but I have no data on this yet. I think these findings may also be relevant to Dr. Howie's comments on the role of a transmissible agent in the aetiology of auto-immune haemolytic anaemia in mice.

Stanley: Did any of the mice revert eventually and become Coombs' positive?

Woodruff: Yes. None of the hybrid animals reverted but two of the mice given syngeneic cells did. What we wanted to find out was *when* they reverted, that is, how long it would take for the new population of lymphoid cells to produce the conditions which give rise to a positive Coombs' test. What I think will happen is that most of the animals will die before showing reversion from Combs' negative to positive. We do not know how many more animals might convert if they lived longer.

Burnet: The very interesting contrast between the two sorts of results reported from Otago by Dr. Howie and from our own institute by Dr. Holmes offers a good example of the virtue of studying the same material in two different laboratories. Drs. Howie and Helyer are to be congratulated on their success in transferring thymus tissue to the (CBA×T6) hybrid. We have always thought that it might be a good idea to do these transplantation experiments in a recipient which would accept parental organs, such as the (NZB×C3H)F_1 hybrid, but I wonder now if this is not the wrong recipient. An F_1 hybrid may have some resistance, whether the way in which auto-immune haemolytic anaemia is produced is by the development of a forbidden clone or through a virus initiating the equivalent of a forbidden clone. There may be advantages in using a completely unrelated animal which has been made immunologically incompetent by the manipulation of neonatal thymectomy.

Another problem here is disagreement about the direct effect of neonatal thymectomy. We did not, unlike some other workers, find any acceleration in the onset of the disease. This difference in results may be related to differences in the bacterial and viral flora in the mouse stocks concerned. The positive results Dr. Holmes is now getting with irradiated and partially protected mice may be similar to the positive results Dr. Howie has reported in the baby mice that he has made immunologically incompetent. I think that both these procedures may have allowed actively pathogenic clones of cells to survive.

Evidence which seems opposed to the hypothesis of a virus aetiology of auto-immune disease in NZB mice is the readiness with which we can transfer Coombs' positiveness to syngeneic young recipients with live cell suspensions; this is quite impossible with killed cells or extracts. The reciprocal transmission of auto-immune disease through both males and females found in all the NZB mice and all the hybrids we have tested is also against a direct vertical transmission, although I agree that this cannot be excluded. Dr. Holmes and Miss Sibthorpe have shown (unpublished material) that there is extremely regular transfer of Coombs' positiveness when spleen cells from a fully developed, Coombs' positive male are transferred to syngeneic recipients. This does suggest that it is the spleen cells themselves that are the active agent. Thymic and blood lymphocytes and lymphocytes from the lymph nodes, in the same doses as the spleen cells, cannot transfer Coombs' positivity in this way. We feel confident that it is the spleen cells that are pathogenic although it is still possible that the changes have been initiated by another process.

The apparent discrepancies in the results from our institute and the group at Otaga are probably associated with technical differences, and it will be an interesting exercise to sort these out.

Howie: I agree with Sir Macfarlane on the whole. It is possible, however, that the actual process of thymectomy has a selective effect on the mice. We have found that the long-survival group of thymectomized CBA/T6 mice have a heavier average weight curve than non-thymectomized mice of this strain, and CBA/T6 mice grafted with auto-immune thymus glands are significantly heavier than intact animals of the same strain. This suggests to me that these thymectomized or grafted animals may have been stronger or more mature

members of their litters in the first place and may have had an initial advantage over their weaker or less mature siblings. This may have enabled them to withstand thymectomy without developing wasting disease. It may also explain why a small proportion of the thymectomized NZB mice have a relatively prolonged survival time compared with the majority which die prematurely with wasting disease.

There are difficulties in relying entirely on the Coombs' test with hybrids. Positive Coombs' tests in the (NZB×NZW) hybrid are commonly evanescent and transitory. Only about 50 per cent of these animals ever show a positive test and yet they all die of lupus nephritis between about 8 and 14 months. Our (CBA/T6×NZB) hybrids have a negative, or barely positive, Coombs' test, and yet, when they die, at about 20 to 24 months, they have the most gross and appalling renal disease. The (CBA/T6×NZB) hybrid has lupus nephritis rather than haemolytic anaemia. This illustrates one of the principal problems of using a single marker. These animals show all sorts of intergrades between haemolytic anaemia and lupus nephritis, and which particular syndrome develops may depend in part on the hybrid that one is using.

Holmes: The operative mortality in our NZB mice thymectomized at four weeks was very low indeed and there was no evidence, certainly in this group nor, I think, in the group thymectomized earlier, that only the potentially stronger mice survived.

Stanley: Two more approaches to this problem could usefully be investigated. The first is study of the NZB mouse in a germ-free state; this would certainly illuminate some of the differences in the results from different laboratories. Neonatal thymectomy under these conditions would be interesting. The second approach would be to apply the techniques of modern virology, particularly those used to study oncogenic viruses such as the SV40 and polyoma viruses and the adenoviruses, in an attempt to detect a non-infectious viral genome. Has any work of this sort been done?

Burnet: Not in our laboratories.

Howie: We have made some desultory attempts to use these techniques. We have introduced material from NZB mice into various groups of weanling mice, thymectomized mice and so on, but there have been no really serious efforts to do much about it.

Nossal: Professor Stanley, can you tell us in biological terms exactly what you think should be done?

Stanley: When the problem of the transformation of a normal cell to a malignant one by an agent such as the SV40 virus was first investigated, it was thought that the transformed cell synthesized a new antigen which was not related to, and did not contain, the complete infective virus. Then A. B. Sabin and M. A. Koch (1963). *Proc. natn. Acad. Sci. U.S.A.*, **49**, 304) exposed these transformed cultured cells to X-irradiation and various types of tissue-culture manipulation and they were able to isolate complete infective virus. They concluded that some of the transformed cells contained the non-infectious viral genome. This is an example of the sort of thing that should be done.

Burnet: I would suggest that the irradiated material should be inoculated into neonatal mice of strains T6 and CBA/T6.

Miller: M. J. de Vries and W. Hijmans (1965. *Communicated to* Information Exchange Group No. 5, Immunopathology) have described a deficiency of thymic epithelium in NZB mice. They have observed that the epithelial cells in the medulla are foetal in type: they are much smaller than normal (about 10 to 12 μ in diameter) with scanty cytoplasm. The band of medullary tissue is much narrower than in normal mice and lacks the "clear areas" normally occupied by groups of epithelial cells. Have Dr. Holmes or Dr. Howie confirmed these observations?

I would also like to ask Dr. Howie if he has studied the regeneration of CBA thymus grafts in NZB mice thymectomized at birth. There may be something in the NZB mouse (possibly a virus) which adversely influences regeneration of the thymic epithelium of the CBA donor.

Howie: Our work to date on normal animals with grafted autoimmune thymuses has been on a basis of survival studies, with terminal killing only. We have studied progress in terms of parameters such as the Coombs' test, haematocrit values and antinuclear tests at intervals during life, but we have not yet electively killed the animals at stages throughout their life. We plan to do this for our next set of experiments.

Burnet: We can confirm de Vries' statement (de Vries and Hijmans, 1965, *loc. cit.*) that the average intensity of epithelialization of the

medulla in adult NZB thymuses is less than we usually see. But in the (NZB×NZW) hybrid it seems to be rather more than usual. I think this means that these observations are not significant.

Holmes: We have been impressed by the increased proliferation of epithelial cells of some of the NZB grafts implanted in irradiated (NZB×T6)F_1 hybrids compared with the normal appearance of the grafts in non-irradiated hybrid hosts.

Good: The histological pictures that Dr. Howie presented looked very like the lesions of lupus erythematosus in man. There is no evidence of a direct antibody assault on the glomerular membrane in this syndrome, but there is an indirect assault in the form of γ-globulin and complement complexes that are laid down in lumpy deposits on the epithelial side of the basement membrane. These findings are similar to Dixon's (Dixon, F. J., *et al.* [1961]. *J. exp. Med.*, **113**, 899) when his rabbits developed chronic renal disease associated with deposits of antigen-antibody and complement complexes. Dr. Howie, have you done any immunohistochemical studies on your lesions to help elucidate their pathogenesis?

Howie: We have done some work with fluorescent labelled anti-globulin on the (NZB×NZW) kidney in the mouse. A considerable amount of fluorescent material is deposited in this kidney. It first appears at about six months in very small quantities and increases to large amounts by the time the haemolytic disorder has developed fully. The fluorescent material shows as irregular linear deposits along the basement membrane.

Good: Just as in human lupus?

Howie: Yes, it is identical with that.

Good: The only syndrome in which we know that there is a direct attack by antibody on the glomerular membrane is the Masugi anti-membrane nephrotoxic serum glomerulonephritis (Masugi, M. [1933]. *Beitr. path. Anat.*, **91**, 82; [1933–34]. *Beitr. path. Anat.*, **92**, 429; Dixon, F. J., *et al.* [1966]. *In* Immunopathology, Proc. IV Int. Symp., in press). In this condition there is a linear deposition of γ-globulin and complement on the basement membrane. In situations in which antigen-antibody complexes are formed, both the γ-globulin, the complement and, where the antigen can be defined, the antigen are deposited in lumpy deposits in the glomerulus. This can be visualized with im-

munohistochemical techniques and by light microscopy of very thin sections.

Burnet: My colleagues, Professor R. C. Nairn and Dr. J. D. Hicks, working independently, (unpublished material) and I have been studying the nephritic lesions in both the NZB mouse and the (NZB × NZW) hybrid. We have found that the glomerular deposit in these mice contains not only γ-globulin, demonstrated by the appropriate immunofluorescence test, but albumen, fibrin and fatty material as well. It looks as if all the components of plasma have escaped into the glomerulus and this makes it difficult to assess the significance of the γ-globulin that is there.

Good: These results are quite different from what we see in human lupus erythematosus in which the immunohistochemical properties of the lesions are absolutely specific. The deposits in human lupus do not contain albumen. I agree that it is hard to interpret the immunohistochemistry in these lesions when there is a general exudation of protein.

Howie: It is important to work with very early changes on these mice. By the time the lesion is well established we are dealing with a grossly damaged glomerulus which may be most misleading.

Feldman: Sir Macfarlane, do the NZB mice, at the peak of their auto-immune manifestation, form γG- and γM-immunoglobulins in response to antigens of extrinsic origin? Do they produce a homograft reaction?

Burnet: These are all good antibody-forming mice.

Kaplan: Sir Macfarlane, have animals that have become Coombs' positive and have other signs of active auto-immune disease been tested for their responsiveness to delayed hypersensitivity antigens?

Burnet: No.

Feldman: You demonstrated, Dr. Howie, that thymectomized CBA/T6 mice implanted with thymuses from NZB or NZB-hybrid donors show positive Coombs' tests, L.E. tests and so on. Did you try to implant the thymuses within diffusion chambers, and, if so, is the Coombs' test also positive under these conditions? This would indicate whether the reactive cells are of donor or host origin.

Howie: We have worked with diffusion chambers and used the techniques described by D. Osoba and J. F. A. P. Miller (1964. *J. exp. Med.*,

119, 177). Our main problem was that most of our animals died with liver necrosis and paraplegia; apparently they were not protected from the thymectomy syndrome. It is impossible to say if the mice developed auto-immune disease, because they died too soon from liver necrosis.

Dameshek: Professor Woodruff has done some rather formidable experiments to make his NZB mice Coombs' negative. I wonder if this effect could be achieved more simply with immunosuppressive drugs such as 6-mercaptopurine or Imuran.

Concerning thymus grafts in Coombs' positive mice, Dr. Howie's interpretation that something positive is added when the graft is irradiated was substantiated by Schwartz (Schwartz, R. S., and Beldotti, L. [1963]. *Science*, **140**, 3563). Schwartz found that when mice which had recovered from a graft-versus-host reaction were irradiated with 800 r. they almost all again developed typical runting. In other words, the clones already present were probably reactivated by irradiation and induced, in some manner, a breakdown in tolerance.

Furth: Spontaneous glomerulonephritis is not uncommon in many strains of mice. Radiation can also induce similar morphological changes. Dr. Howie, do you think that the picture you showed is truly that of lupus, or could it be some sort of glomerulonephritis or nephrosclerosis that occurs in irradiated mice?

Howie: We think that the renal changes we have observed closely reproduce the changes found in human lupus erythematosus. Our findings have been confirmed by Drs. C. L. Pirani and V. E. Pollak in Chicago (personal communication). It is difficult to say how exactly these changes reproduce the features of human lupus, and perhaps I should be more specific about them. The lesions start as a proliferation of the endothelium and this is the primary reaction. It is succeeded by changes in the basement membrane and other changes all of which we believe to be secondary. This early primary endothelial proliferation coincides with the first evidence of immunofluorescence.

Mackay: I do not think there could be any doubt in the minds of those who see both human lupus erythematosus and murine auto-immune lesions that the renal lesions of the mice are as near as one can get to the lesions of human lupus nephritis. The ultimate requirement for the diagnosis of lupus in man is the presence of haematoxylin bodies.

Dr. Howie, are these present in the kidneys of your mice? Would you also be prepared to say whether you think the disease in your mice is best explained by a virus-induced transformation of lymphoid cells in the thymus to forbidden clones, which then produce the systemic features of the disease?

Howie: There is no evidence for this either way and I am not prepared to commit myself about it! We have occasionally imagined that we have seen haematoxylin bodies but I am not prepared to pontificate about these either!

IMMUNOCYTES AND IMMUNOPROLIFERATIVE DISORDERS

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THE immune response is basically a cellular one in which a highly specialized group of cells, immunocytes, reacts to foreign or "not-self" materials, antigens. Included in this response to antigen are macrophages (histiocytes or reticulo-endothelial cells), lymphocytes, plasma cells and a primitive cell I have called the immunoblast (Dameshek, 1963*a*). Although the cellular reaction is often intense it is nevertheless a controlled and self-limited one. In contrast, in such conditions as chronic lymphocytic leukaemia, multiple myeloma and Hodgkin's disease, immunocytes proliferate in an uncontrolled and self-perpetuating manner often in association with immunological abnormalities of various types. The discussion which follows attempts to define this group of conditions which may be called the immunoproliferative disorders, to relate them to the various features of the immune response and to suggest the possibility that these conditions are actually aberrant forms of the immune proliferative reaction.

THE IMMUNOCYTE COMPLEX

The cells concerned in the immune proliferative response have been studied by many groups. In the past quarter of a century emphasis has shifted from the histiocyte as the central immunocyte to the plasma cell and, more recently, to the small lymphocyte (Waksman, 1965). The phagocytic function of the histiocyte is well known. It is presumed that in this cell phagocytosed material containing antigen is prepared for suitable exposure to other cells which eventually synthesize antibodies (Gill and Cole,

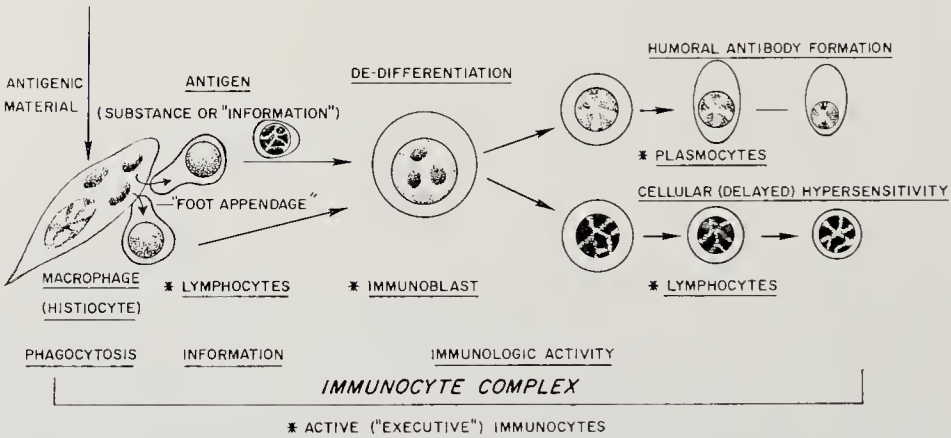

FIG. 1. Immunocyte complex and active immunocytes.

1965) (Fig. 1). Studies by McFarland and Heilman (1965) have demonstrated the gathering of lymphocytes about histiocytes containing phagocytozed material, with pseudopods or "foot appendages" of the lymphocytes evidently making prolonged contact with the histiocyte during the first phase of the immune response. Conceivably, specific information or perhaps actual antigens might be transferred in this way from histiocyte to lymphocytes. Although antigens have been clearly localized within phagocytic cells, they have not yet been visualized within the lymphocytes or other cells synthesizing antibodies.

Antigen has been shown to give rise to the dedifferentiation of

some small lymphocytes to large primitive-looking cells with deep blue, pyroninophilic cytoplasm (Leduc, Coons and Connolly, 1955) (Fig. 2). These large cells have been variously called haemocytoblasts, large pyroninophilic cells, blast forms or immunoblasts. They may have two modes of differentiation, perhaps dependent upon the type of antigen: they may either

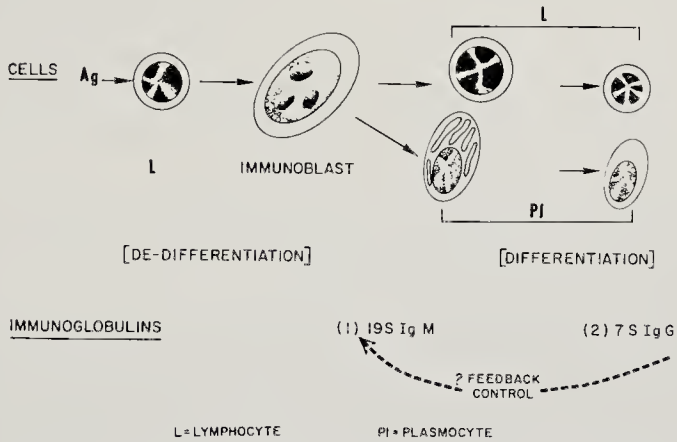

FIG. 2. Active immunocytes and the immunoglobulins. It is implied, but not proved, that γM-immunoglobulin is produced by the immunoblasts and lymphocytes. γG-immunoglobulin is undoubtedly produced by plasma cells. A feedback mechanism controlling the "shut-off" of γM when sufficient γG is produced in the immune response has been postulated (Sahiar and Schwartz, 1964). 19S IgM = γM-immunoglobulin. 7S IgG = γG-immunoglobulin. L = lymphocyte. Pl = plasmacyte.

become lymphocytes or develop into plasma cells. Whether differentiation occurs *via* different blast forms (lymphoblasts, plasmablasts) or from one (immunoblast) is uncertain. In delayed or cellular hypersensitivity the preponderant proliferative response is lymphoid, whereas in the humoral antibody response, at least in its later phases, plasmacytosis is marked (Mellors and Korngold, 1963). The exact derivation of the plasmacyte has yet to be determined. Thus the immune response is characterized

first by the dedifferentiation of lymphocytes to immunoblasts, and second, by a well-defined proliferation, which may be predominantly lymphocytic or plasmacytic.

Of the cells concerned in the immune reaction only the plasmacytes have been proved to be involved in the production of γ-globulin, that is, antibody. The immunoblast, with its large concentration of ribosomes, may make the first in the normal sequence of immunoglobulins (γM-immunoglobulin) in immune responses. It is not known if lymphocytes contribute to immunoglobulin synthesis and a demonstration of this has thus far been lacking. The presence of masses of small lymphocytes about a transplant undergoing rejection (Billingham and Silvers, 1963) as well as in some other immune reactions (for example, tuberculin hypersensitivity) strongly suggests that these cells are active immunocytes.

Sahiar and Schwartz (1964) have demonstrated experimentally an interesting feed-back response which may dictate to some extent the manner of the humoral immune response. γM-immunoglobulin usually appeared as the initial response to administered antigen, followed shortly by increasing quantities of γG-immunoglobulin. The administration of an antipurine drug (6-mercaptopurine) at the same time as antigen could block the development of the γG phase and result in the continued production of peak quantities of γM. If γG-immunoglobulin was injected at this time the amount of γM dropped sharply and γG increased. This indicated a possible feed-back sequence in the immune response: the development of a sufficient quantity of γG apparently led to the shut off of further γG production. The cellular basis for the initial γM response to antigen remains uncertain, although the γG response which follows is clearly plasmacytic in nature.

Since lymphocytes, plasmacytes and immunoblasts are actively engaged in the immune response they may be termed not only immunologically competent (Medawar, 1964) but "executive

cells" as well.† Other cells possibly concerned in the immunological response are: the *eosinophil* (Litt, 1964), which is often found at the site of antigen-antibody interaction but has an uncertain role; and the *mast cell* which contains heparin, histamine and serotonin and may be important in certain types of immune responses (Mota, 1963), for example, anaphylaxis.

The place of the thymus in relation to the immunocyte complex is still uncertain, even after several years of intensive investigation. The mammalian thymus is of great importance in the development of peripheral lymphoid tissues (Miller, 1964). In steady-state lymphoid tissue the thymus may supply lymph nodes and spleen with new lymphocytes. These cells either develop immunological competence when they reach the lymph nodes and spleen or have already acquired it in the thymus. The thymus also appears to secrete a humoral factor of importance in lymphopoiesis and in the functional (immunological) maturation of lymphoid tissues (Osoba and Miller, 1964). According to Peterson, Cooper and Good (1965), the thymus may direct the growth of the collar of lymphocytes about the germinal centre, whereas the bursa of Fabricius in the chicken, or its analogue in mammals (? appendix or tonsils), may control the activity of the germinal centre. That the thymus supplies lymphocytes to the rest of the body is clear; that it conditions or educates them during their continued recirculation in the lymph nodes, thoracic duct, thymus and lymphatic channels is not certain (Ford and Micklem, 1963).

LEUKAEMIAS AND RELATED STATES CONCERNED WITH THE PROLIFERATIONS OF IMMUNOCYTES

Several forms of neoplasia of the leucocytic tissues, whether localized (sarcoma) or generalized (leukaemia) are characterized

† The term immunologically competent cell is excellently conceived but represents "a bit of a mouthful" (Medawar, 1964). I propose to use the word "immunocyte" in place of this term. This is short; it also adds a functional dimension to the expression as it refers to a cell which is immunologically competent in the present and may also be actively engaged in the immune proliferative process in the future.

by (a) a self-perpetuating proliferation of immunocytes and (b) the presence of various protein or immunological abnormalities. To these neoplastic conditions a few other disorders of indeterminate type may be added, including infectious mononucleosis, Hodgkin's disease and sarcoidosis. These immunoproliferative disorders may be classified as shown in Table I.

TABLE I

THE IMMUNOPROLIFERATIVE DISORDERS

Lymphoproliferative
- Infectious mononucleosis
- Chronic lymphocytic leukaemia
- Lymphosarcomatosis
- Macroglobulinaemia
- Heavy chain disease

Plasmaproliferative
- Multiple myeloma
  - including light chain disease

Reticuloproliferative
- Hodgkin's disease
- Sarcoidosis

With the outstanding exception of multiple myeloma, autoimmune abnormalities figure prominently in these disorders which occur chiefly when the lymphoid system is extensively involved.

THE LYMPHOPROLIFERATIVE DISORDERS

These represent a variable group of conditions: some of them acute, others chronic; some reactive or self-limited others apparently permanent or self-perpetuating; some apparently localized, at least initially, and others generalized. They are all characterized by an abnormal proliferation of lymphoid cells and by the frequent presence of some form of immune abnormality, particularly when the process is generalized, as in leukaemia or in generalized lymphosarcomatosis. In both chronic

lymphocytic leukaemia (Bernard, Geraldes and Boiron, 1964) and Hodgkin's disease (Aisenberg, 1965), an abnormality in the reaction to phytohaemagglutinin has been found indicating a possible disturbance in the dedifferentiation of lymphocytes to immunoblasts.

Acute, self-limited: Of the many conditions in which a well-defined acute disturbance characterized by lymphoid proliferation and abnormal proteins or antibodies is found, infectious mononucleosis is most striking. Here, numerous antibodies may be present: heteroagglutinins, isoagglutinins, positive serological test for syphilis (biologically false positive), cold haemagglutinins and so on. At times auto-immune disturbances including haemolytic anaemia (Green and Goldenberg, 1960) and idiopathic thrombocytopenic purpura (Grossman and Wolff, 1959) are found. The strikingly abnormal lymphoproliferative response in this disease, consisting of primitive cells closely resembling immunoblasts and of various kinds of abnormal-looking lymphoid cells actively synthesizing DNA (MacKinney, 1965), has many of the histological appearances of the immune response.

Chronic, self-perpetuating: Chronic lymphocytic leukaemia, disseminated lymphosarcomatosis, macroglobulinaemia, and the newly described disorder "heavy chain" disease (Franklin *et al.*, 1963), are characterized by generalized lymphoid proliferation with the accumulation of large groups of lymphocytes in all the tissues, a variable course usually of several years, a variable degree of lymphocytosis in blood and bone marrow, and the common finding of various types of abnormalities of the immune mechanism. These include: hypogammaglobulinaemia and, at times, hypergammaglobulinaemia; a variable degree of anergy; the presence, at times, of cryoglobulins, cold haemagglutinins and, rarely, of well-defined macroglobulins; and various auto-immune disorders including haemolytic anaemia, idiopathic thrombocytopenic purpura, rheumatoid arthritis, Hashimoto's thyroiditis, and vasculitis. The auto-immune disturbances may occur either

before macroglobulinaemia (Ritzman and Levin, 1961), at the same time as the disease is first diagnosed, or not until its later manifestations.

THE PLASMAPROLIFERATIVE DISORDERS

Multiple myeloma: This disorder is characterized by the continued proliferation of plasma cells which gradually accumulate in huge numbers particularly in the bone marrow. Chiefly responsible for the multiplicity of symptoms and for the various laboratory abnormalities is the hypergammaglobulinaemia which is usually of the γG, but occasionally of the γA variety (Osserman and Takatsuki, 1963). Although the globulin has all the characteristics of an individual, highly specific immunoglobulin, no relationship of this presumed antibody to antigen has yet been demonstrated and auto-immune disturbances are conspicuously lacking. This is also the case in what might be called the "light chain" variant of multiple myeloma in which the intense plasmacytic proliferation seems to produce only light chains which are excreted in the urine and there are identified as Bence-Jones protein.

THE RETICULOPROLIFERATIVE DISORDERS

Hodgkin's disease: This condition, whose neoplastic nature has been controversial almost since its discovery, is characterized by a pleomorphic proliferation of lymphocytes and reticulum cells with a variable number of more or less characteristic giant cells. Attempts to incriminate various infections and viruses as aetiological agents have not been successful and the pathogenesis of the disorder remains obscure. Anergy is commonly found (Aisenberg, 1962). Since this abnormality may be found early in the course of the disease the possibility of its presence before the disease is clinically evident must be considered. A few cases

of Hodgkin's disease show auto-immune manifestations including haemolytic anaemia, idiopathic thrombocytopenic purpura and, possibly, Sjögren's syndrome (Talal and Bunim, 1964).

Sarcoidosis: This disorder possesses certain resemblances to Hodgkin's disease such as generalized lymphadenopathy, reticulum-cell proliferation with giant cells, and anergy, but specific immunological abnormalities are uncommon although diffuse hypergammaglobulinaemia is usually found (Kunkel, Simon and Fudenberg, 1958).

THE PROTEIN AND IMMUNOLOGICAL ABNORMALITIES OF THE IMMUNOPROLIFERATIVE DISORDERS

The protein and immunological abnormalities found in the immunoproliferative disorders are both extraordinarily common and extraordinarily diverse. They comprise changes in the total and the various types of globulins, the presence of abnormal antibodies—hetero-, iso-, and auto- in type—, immunological deficiency characterized by alteration in antibody response or a reduction in delayed hypersensitivity, even anergy, and, from the clinical standpoint, a high incidence of various auto-immune disorders.

Waldenström (1962) has used the term monoclonal gammopathy to define the hyperglobulinaemias in which a clearly defined, narrow band of globulin is found. The implication, and this seems a reasonable one, is that the single spike of immunoglobulin is produced by a single clone—however large—of immunocytes, all having the same ancestry. Multiple myeloma, macroglobulinaemia, certain cases of chronic lymphocytic leukaemia, heavy chain disease, and certain examples of dysgammaglobulinaemia fall into this category. Of particular interest are cases of multiple myeloma, first showing a small but sharp spike of γG-immunoglobulin which later becomes larger. This change is followed eventually by plasmacytosis in the bone

marrow and the characteristic osteolytic changes. This sequence clearly indicates an expanding mass or clone of plasma cells (Osserman, 1959).

Specificity of immunoglobulin in auto-immune haemolytic anaemia has been observed in the reaction of anti-red-cell antibodies with various Rh antigens, notably of the "e" type. Leddy and Bakemeier (1964) have demonstrated a striking chemical homogeneity in certain red cell auto-antibodies which resembles the homogeneity of myeloma globulins. This is strong evidence that at least some of these auto-antibodies, with or without overt leukaemic manifestations, may be derived from the immunological activities of a single abnormal clone of immunocytes.

AUTO-IMMUNE DISEASE AND THE IMMUNOPROLIFERATIVE DISORDERS: SOME INFERENCES

A small but increasing number of apparently idiopathic auto-immune disorders eventually terminate in leukaemia, lymphosarcoma, or in reticulum-cell sarcoma. In these cases no clinical, histological or other evidence of leukaemia or lymphosarcoma may be found for several years before the development of the neoplastic process. The usual interpretation is that the auto-immune disturbance is symptomatic of the underlying malignancy. On the other hand, it is possible that the auto-immune disease may exist *before* the neoplastic process becomes apparent and may be closely connected with it. One must consider the possibility that the leukaemia is either an extension of the auto-immune process, or that both the latter and the leukaemia are simply different expressions of the same fundamental disorder.

The development of auto-immunity (an immune reaction against self-components) can either be due to immunological reactivity (a) with unrecognized (previously sequestered) or altered self-antigens or (b) with *normal* self-antigens reacting with *abnormal* self-immunocytes. Evidence for the latter

concept has been obtained in mice in the graft-versus-host reaction (Oliner, Schwartz and Dameshek, 1961). By the injection of parental spleen cells into F_1 hybrids, an illness is produced which terminates in wasting or runting. Haematological and immunological studies in these animals have demonstrated various auto-immune disturbances which seem clearly to be due to a reaction of the grafted genetically-different, and thus abnormal, immunocytes against the host.

The concept of the abnormal or forbidden clone of immunocytes, initially tolerant of the host-patient, but later reacting against him to cause auto-immune disease, has been emphasized by several workers (Burnet, 1959; Dameshek, 1963*b*). The abnormal clone could be transplacentally transferred, could arise by mutation, or could be genetically derived. The genetic disorders of auto-immune haemolytic anaemia and systemic lupus erythematosus in New Zealand mice (Bielchowsky, Helyer and Howie, 1959) support the last of these possibilities. It is conceivable that tolerance for host antigens may be lost by the abnormal clone as the result of triggering factors (ionizing radiation, sunlight, cytotoxic chemicals and so on). It is also possible that the forbidden clone supposedly responsible for the auto-immune disease may enlarge by continuous proliferation in response to the continuous stimulation of self-antigens. If this expansion becomes sufficiently great, clinical evidence of it in the form of lymphosarcoma, Hodgkin's disease or leukaemia might appear. We have observed cases of auto-immune haemolytic anaemia eventually developing chronic lymphocytic leukaemia, cases of cold haemagglutinin disease with an auto-immune haemolytic anaemia preceding the development of macroglobulinaemia, auto-immune haemolytic anaemia progressing to the Sjögren-Mikulicz syndrome and finally showing localized masses of lymphosarcoma, typical Sjögren's disease ultimately showing generalized lymphoproliferative disease and so on.

We can infer from such cases that, at least in some instances,

auto-immunity and certain forms of leukaemia, especially the lymphocytic variety, are fundamentally either the same disease or different expressions of the same disturbance. The leukaemic disease is quantitatively more striking and widely generalized and it is invasive of numerous tissues and so more damaging. One may speculate that in both auto-immune disease and leukaemia an abnormal clone of immunocytes has developed, by mutation or other mechanisms. The clone becomes increasingly larger as self-antigens, coming to it, are not recognized by the abnormal immunocytes, which proliferate. This may result in an abnormal immune response (auto-immunity), in a localized (lymphosarcomatous) process, or in generalized (leukaemic) disease.

EXPERIMENTAL EVIDENCE

Experimental evidence for some of these speculations has recently been obtained by Schwartz and Beldotti (1965) and Mellors (1966). Schwartz noted that some animals apparently recovering from the auto-immune abnormalities of graft-versus-host disease eventually developed a generalized proliferative disease of lymphoid and reticulum-cell tissue. Histologically this neoplasm, which was malignant and invasive, was pleomorphic consisting of both lymphoid and reticulum cells, and with giant cells resembling the Reed-Sternberg cells seen in Hodgkin's disease. Mellors has demonstrated that some NZB mice, with their genetically determined auto-immune haemolytic anaemia, develop a terminal pleomorphic generalized lymphomatous or leukaemic condition which can be transplanted. That the neoplasms in both these experimental situations follow an immunological disease was clear, but whether they are actually the end result of the immunological disease or due to other factors, for example a virus, is yet to be demonstrated. It can be stated that the self-perpetuating immunological disorder in these examples eventually leads to a leukaemic condition. The leukaemic disease in this

context could be interpreted as an extension of a self-perpetuating auto-immune proliferation.

This theme has been advanced in various forms by Green (1954), Kaplan and Smithers (1959), Tyler (1962) and Dameshek and Schwartz (1959). Tyler postulated that an immunologically competent cell, somewhat different genetically from the remainder of the host's antigens, would proliferate constantly in response to them and become leukaemic. Kaplan and Smithers were the first to suggest that graft-versus-host disease might be an analogue of Hodgkin's disease. Metcalf's experiments dealing with antigenic bombardment and the eventual development of a neoplastic disease of immunocytes are in line with this concept (Metcalf, 1961).

Simple overproduction of abnormal immunocytes in response to self-antigens could be one mechanism for immunoproliferative disease, an error of normal immunological proliferation could be another. Normal immunoglobulin production, which is a two-step process—first γM- and then γG-immunoglobulins are produced—may be altered by an antimetabolite such as 6-mercaptopurine, with the result that the first step (macroglobulin production) becomes continuous, with a greatly reduced second or γG phase. It is conceivable that such clinical conditions as macroglobulinaemia and multiple myeloma may be based on some such error of normal immunological proliferation, perhaps induced by a chemical, viral or other insult.

As well as human experiments of nature in which auto-immune disease terminates as leukaemic disease, there are at least two important animal analogues: Aleutian mink disease and the disease of the NZB mice with auto-immune haemolytic anaemia. In the former—a virus-mediated disease—there is considerable plasmacytosis of the bone marrow and a diffuse hyperglobulinaemia (Obel, 1959). Eventually, in approximately 10 per cent of the animals, the diffuse globulin curve, shown on electrophoresis, becomes a sharp monoclonal spike (Porter, Dixon and Larson,

1965); simultaneously the marrow shows a striking plasmacytosis characteristic of multiple myeloma, and Bence-Jones proteinuria develops. Although the exact interpretation of these events may not be clear, it seems evident that here, too, an essentially immunological proliferation, induced by a virus, becomes a neoplastic immunoproliferative disorder, that is, multiple myeloma. Mellors' description of the development of lymphocytic leukaemia in NZB mice which previously had shown the usual genetically determined auto-immune haemolytic anaemia has already been cited.

Some forms of leukaemia and related diseases may be, if not immunologically mediated, at least immunologically associated. The leukaemic proliferation might be considered as a reaction of abnormal immunocytes to self-antigens which although immunological (auto-immune) in design eventually becomes leukaemic in scope.

This hypothesis is contrary to the contention of many virologists and others that viruses mediate or bring about all leukaemic proliferations. Whether it is essential to hold to a single, univalent concept for the aetiology of leukaemia or whether polyvalent aetiological mechanisms occur is debatable. Viruses may initiate an immunological abnormality but they are not necessarily responsible for all that come after. It is possible that in at least some of the chronic immunoproliferative disorders an abnormal clone of immunocytes, perhaps initially virus-mediated, may at first result in occult auto-immune disease (that is, a persistently positive serological abnormality), then in overt auto-immune disease and finally in a clinically discernible mass of proliferating immunocytes, for example leukaemia, Hodgkin's disease or multiple myeloma.

SUMMARY

In previous reports we have emphasized the frequent occurrence of immunological abnormalities, notably of the auto-

immune type, in chronic lymphocytic leukaemia and in the lymphoproliferative disorders in general. We have commented on the many relationships and similarities between certain forms of auto-immunity and of leukaemia, and on the often similar therapeutic approaches to these disorders. In the present discussion an attempt has been made to extend this relationship to an actual identity between certain forms of abnormal immunological proliferation and such neoplastic disturbances as chronic lymphocytic leukaemia, multiple myeloma and Hodgkin's disease. We have envisaged a system of immunocytes which has the particular capacity to react with antigenic materials. This reaction, which is ordinarily a self-limited one, may, under abnormal circumstances, become self-reactive and self-perpetuating. Auto-immunity may result and if continued and expanded may produce a greatly expanded proliferation of cells, as in leukaemia, lymphosarcoma, multiple myeloma and Hodgkin's disease. In this context auto-immunity and some forms of leukaemia may be regarded as different expressions of the same abnormal immunoproliferative reaction.

It is suggested that certain forms of leukaemia may develop as the result of continued proliferation of an abnormal clone of immunocytes, with the appearance of specific immunological abnormalities. From clinical and experimental observations an attempt has been made to categorize a group of immunoproliferative disorders with two chief characteristics: (a) self-perpetuating proliferations of the various forms of immunocytes and (b) abnormal immunological reactions.

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THE THYMUS AND HUMAN DISEASES WITH AUTO-IMMUNE CONCOMITANTS, WITH SPECIAL REFERENCE TO MYASTHENIA GRAVIS

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Thymic abnormalities in man, as revealed by X-ray, surgery or post-mortem gross and microscopic examinations, have been observed in association with several clinical disorders. These conditions have been reviewed by Miller, Marshall and White (1962) and Miller (1963). Probably the most frequent association is between thymic "hyperplasia"† with the formation of germinal centres in the thymic medulla and the neuromuscular disorder myasthenia gravis (Sloan, 1943; Collins and Bratton, 1946; Castleman and Norris, 1949; Ringertz, 1951; Castleman, 1955). Similar histopathological changes have been less frequently described in autopsy material from individuals without apparent chronic illnesses, and in some with thyrotoxicosis, Addison's disease and acromegaly (Sloan, 1943); in association with rheumatoid arthritis (Burnet and Mackay, 1962); and in patients with systemic lupus erythematosus (Mackay and de Gail, 1963; Alarcòn-Segovia *et al.*, 1963; Hutchins and Harvey, 1964; Burnet and Mackay, 1965). Thymomas, which are clearly neoplastic growths with several different morphological charac-

† The weight and gross appearance of the gland may be within normal limits; the term hyperplasia refers to microscopic morphology only.

teristics, have been observed in association with myasthenia gravis (Weigert, 1901; Starr, 1912; Bell, 1917; Sloan, 1943; Castleman and Norris, 1949; Ringertz, 1951; Castleman, 1955); "acquired" agammaglobulinaemia (Good and Varco, 1955; Ramos, 1956; and others); hypergammaglobulinaemia with purpura (Birch *et al.*, 1964); aregenerative anaemia with and without associated leucopenia and thrombocytopenia (Radojević and Hahn, 1935; and others. See also the reviews by Havard and Bodley Scott, 1960, and Roland, 1964); acute auto-immune haemolytic anaemia (Meyer and Bertcher, 1960); Sjögren's syndrome, rheumatoid arthritis and scleroderma (Lattes, 1962). Thymomas have been recorded in a patient with cutaneous lesions of the pemphigus vulgaris type (Kough and Barnes, 1964); in systemic lupus erythematosus (Larsson, 1963); and in Cushing's syndrome (Leyton, Turnbull and Bratton, 1931; Kepler, 1933; Hubble, 1949; Castleman, 1955). Histopathological change in the thymus may be seen in Hodgkin's disease (reviewed by Thomson, 1955) and in acute leukaemia. Absence of the thymus, an undescended thymus or a thymus resembling its embryonic epithelial primordium before lymphoid induction have been observed in certain of the hereditary immunological deficiency states in man, for example, the Swiss type of agammaglobulinaemia and hypogammaglobulinaemia associated with ataxia telangiectasia (reviewed by Good *et al.*, 1965).

The precise functional relationships between these syndromes and the associated thymic pathology still remain largely unelucidated. The clear demonstration of substantial immunological derangement or serological auto-immune concomitants in several of the above conditions and the independently emerging evidence that the thymus has a significant and unique role in the development of immunological competence (Good *et al.*, 1965; Miller, 1963) constitute circumstantial evidence for the presence of functional immune relationships between thymic changes and several clinical abnormalities.

Our own investigations have dealt with certain serological phenomena that occur with myasthenia gravis, are tentatively classed as auto-immune and show a striking correlation with the presence of thymic pathology, especially thymomas.

The hypothesis advanced independently by Nastuk, Plescia and Osserman (1960) and Simpson (1960) that myasthenia gravis may be a disorder with an auto-immune aetiology and early studies dealing with the probable immunological associations of myasthenia gravis have recently been reviewed in detail by Strauss and co-workers (1965). They need not be considered here.

At the 1964 New York Academy of Sciences' Conference on Auto-immunity, Strauss and co-workers (1965) reported that the serum γ-globulins of 30 per cent of patients with myasthenia gravis (99 out of 336) reacted concurrently *in vitro* against alternate skeletal muscle striations (A-bands) and thymic epithelial cell cytoplasm as demonstrated by indirect fluorescent antibody techniques. Serum from 19 out of 20 patients with myasthenia gravis and *associated thymomas* (95 per cent) was reactive against both skeletal muscle and thymus. These findings essentially confirmed and extended the observations of earlier workers (Strauss *et al.*, 1960; Beutner *et al.*, 1962; Feltkamp, van der Geld and Oosterhuis, 1963; van der Geld and Oosterhuis, 1963; van der Geld *et al.*, 1963) on the presence of a muscle-binding globulin factor in myasthenia gravis. They also supported the more recent finding (van der Geld, Feltkamp and Oosterhuis, 1964) of associated serological cross-reactivity with thymic epithelial cells. In the same coded and randomized survey (Strauss *et al.*, 1965), serological reactivity against both skeletal muscle and thymus was not seen within test limits in the sera of 128 out of 129 healthy individuals and 673 out of 674 disease-control individuals. These included patients with other so-called auto-immune diseases—myopathies, dystrophies, heredo-familial neuro-muscular diseases, lymphomas, tuberculosis, granulomatoses,

FIG. 1a. Indirect immunofluorescence observations. Pattern observed with serum from 930 individuals in the earlier series (Strauss *et al.*, 1965): 223 patients with myasthenia gravis, 128 normal controls and 579 disease controls. The same pattern was observed in the present study with serum from 32 patients with thymomas *not associated* with myasthenia gravis. No fluorescent striations nor thymic epithelial cells were seen. (× 900). mu = muscle. thy = thymus.

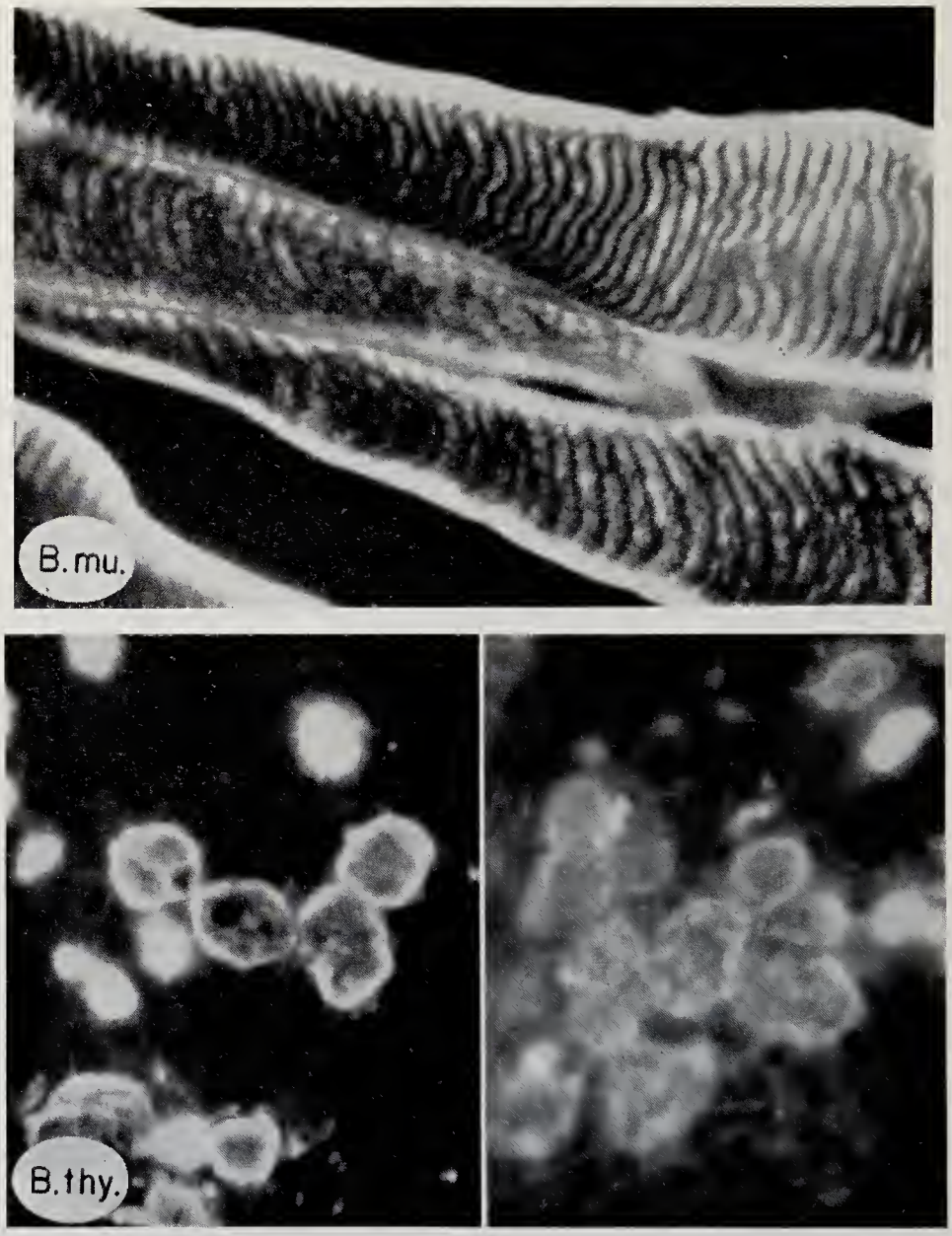


FIG. 1b. Pattern of concurrent immunofluorescence in alternate skeletal muscle striations and thymic epithelial cells seen in the earlier series (Strauss *et al.*, 1965): serum from 93 patients with myasthenia gravis, one normal individual and one patient with a thymoma associated with aregenerative anaemia (no myasthenia gravis). In the present study the same pattern was produced by serum from 7 patients with thymomas *unassociated* with myasthenia gravis. Serum from 12 out of 13 additional patients with thymomas and myasthenia gravis, newly studied, also produced this staining pattern. The cell membranes and granular cytoplasmic material of the thymic epithelial cells are intensely fluorescent. (× 900). mu = muscle. thy = thymus.

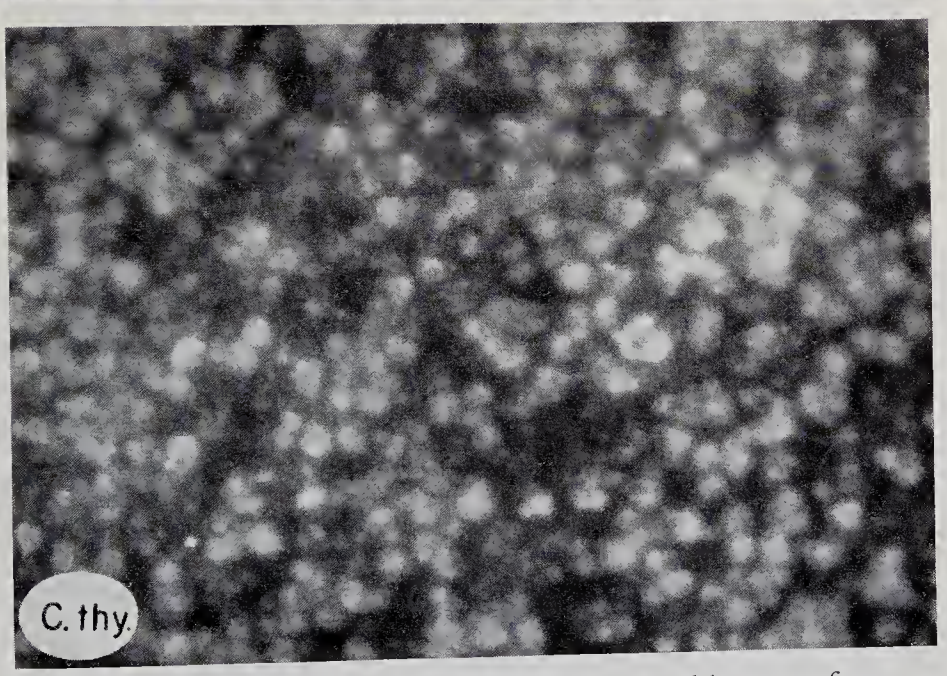
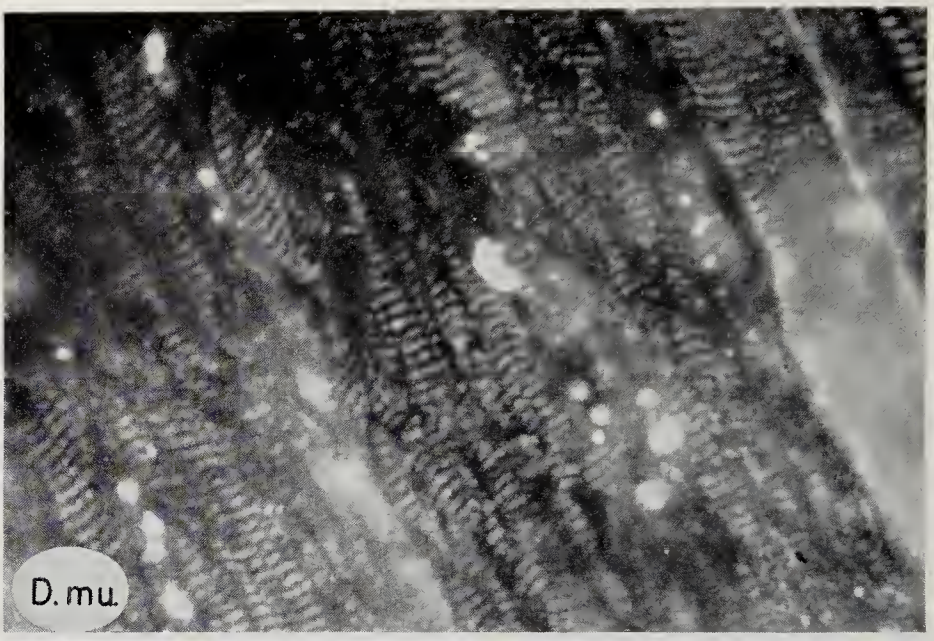


FIG. 1c. Pattern of nuclear immunofluorescence produced by serum from 105 patients: 64 with systemic lupus erythematosus, 11 with rheumatoid arthritis, 2 with scleroderma and 14 with myasthenia gravis (earlier series, Strauss *et al.*, 1965). The same patterns were produced in the present study by serum from 7 patients with thymomas *unassociated* with myasthenia gravis. (×900). mu= muscle. thy=thymus.



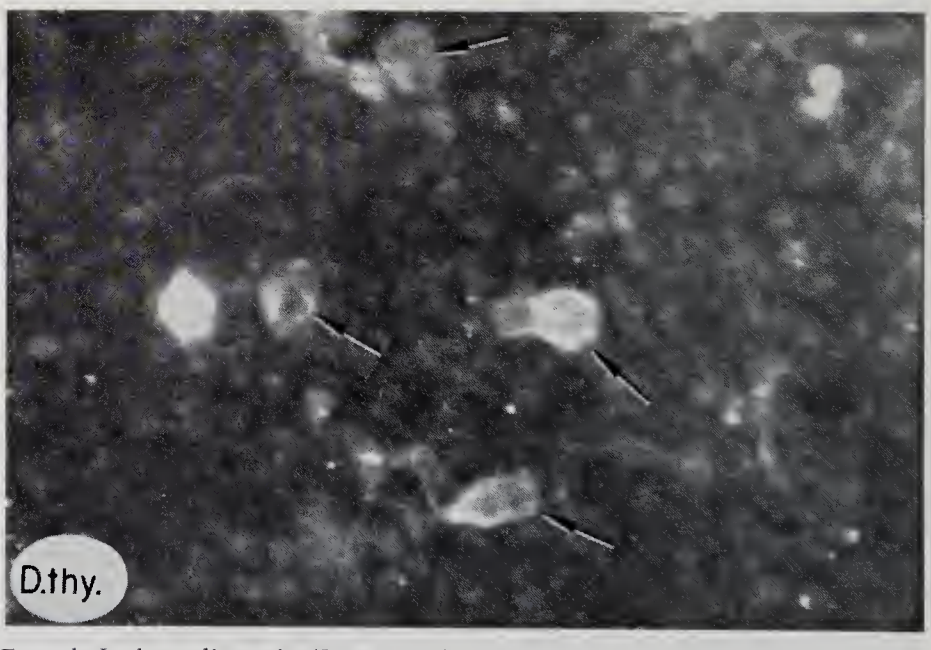


FIG. 1d. In the earlier series (Strauss *et al.*, 1965) serum from 6 patients with myasthenia gravis produced both antinuclear and concomitant antistriational, antithymic epithelial cell reactions. The same reaction pattern was demonstrated in the present series with serum from 5 patients with thymomas *unassociated* with myasthenia gravis. In this group reactivity with thymic epithelial cells was obscured by the diffuse reaction with thymocyte nuclei at the 1:60 screening dilution. At dilutions of 1:120, 1:240 and higher, antinuclear reactions diminished but fluorescence in the epithelial cell cytoplasm persisted and was readily revealed. Note thymic epithelial cells against faint background of fluorescent thymocyte nuclei (arrows) at serum dilution 1:240. (× 900). mu = muscle. thy = thymus.

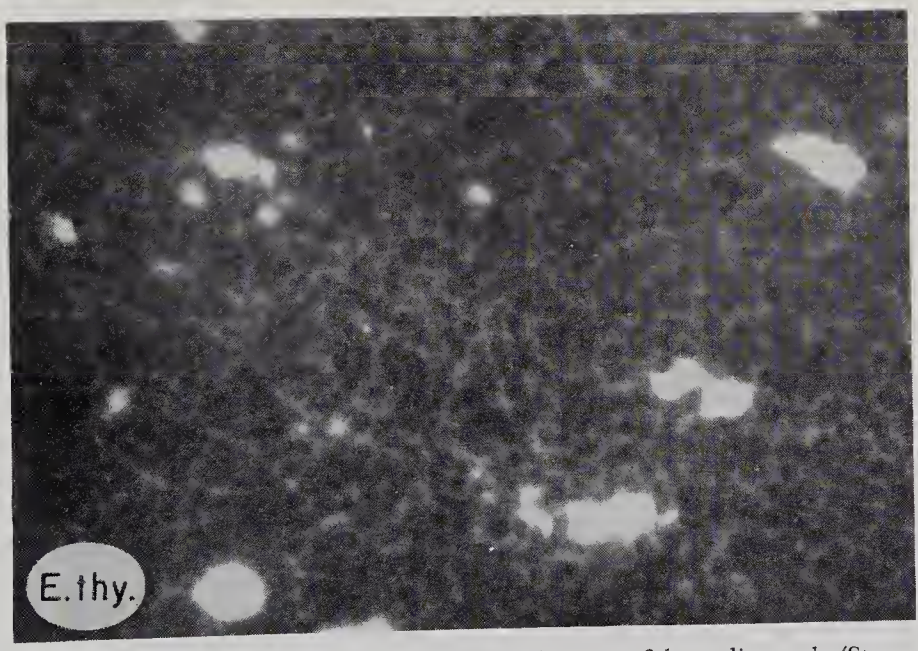


FIG. 1e. Three individuals in the disease control group of the earlier study (Strauss *et al.*, 1965) had serum which reacted with alternate skeletal muscle striations at a serum dilution of 1 : 60; serum did not react with thymic epithelial cell cytoplasm. At the next highest serum dilution—1 : 120—the same serum failed to react with skeletal muscle striations. In the present study no such reaction patterns were encountered. No non-concurrent thymic epithelial cell reactions have been observed to date. (× 900). mu = muscle, thy = thymus.

dysproteinaemias, thymomas unassociated with myasthenia gravis and so on. Nor was serological reactivity present in individuals with histories of anticholinesterase consumption in the absence of myasthenia gravis (Table I). Concurrent reactivities in the serum were observed in one normal control and in a patient with aregenerative anaemia and associated thymoma.

TABLE I

DISEASE-CONTROL SERIES: THE NUMBER OF PATIENTS AND THEIR DIAGNOSES†

(Strauss *et al.*, 1965)

Systemic lupus erythematosus (67), Rheumatoid arthritis (30), Sjögren's syndrome (19), Dermatomyositis (20), Scleroderma (12), Hashimoto's thyroiditis (6), Unclassified collagen vascular disease (7), Auto-immune haemolytic anaemia (3), Post-cardiotomy syndrome (3), Chronic hepatitis (3), Nephrotic syndrome (3), Chronic glomerulonephritis (3), Polymyositis—Inflammatory myopathy (35), Idiopathic myopathy (22), Carcinomatous myopathy (3), Endocrinopathic myopathy (8), Steroid myopathy (3), Sarcoid myopathy (3), Pseudohypertrophic muscular dystrophy (26), Facioscapulohumeral muscular dystrophy (5), Unclassified progressive muscular dystrophy (27), Myotonic dystrophy (35), Peroneal muscular atrophy (18), Amyotrophic lateral sclerosis (36), Familial periodic paralysis (3), Paroxysmal myoglobinuria (3), Multiple sclerosis (6), Friedreich's ataxia (3), Polyneuropathy (10), Multiple myeloma (13), Waldenström macroglobulinaemia (3), Lymphosarcoma (3), Mycosis fungoides (7), Hodgkin's disease (3), Thymoma without associated myasthenia gravis (3), Tuberculosis (41), Hansen's Disease (3), Boeck's sarcoid (4), Whipple's disease (3), Non-myasthenic individuals with a history of chronic anticholinesterase consumption (11), Status postirradiation for status thymolymphaticus (28), Diabetes mellitus (8), Miscellaneous other disorders (122).

† Serum from an additional 211 patients with assorted neoplastic disorders other than thymomas have also been studied; no reactions with muscle striations and thymic epithelial cells have been observed in this group.

The patterns of immunofluorescence staining encountered in these studies, as well as in the supplemental studies are illustrated in Figs. 1a–e (Strauss *et al.*, 1966).

EXTENDED STUDY OF SERUM FROM PATIENTS WITH THYMOMAS NOT ASSOCIATED WITH MYASTHENIA GRAVIS

The demonstration of reactivity against striated muscle and thymic epithelial cell cytoplasm in the serum γ-globulins of a

patient with thymoma *unassociated with myasthenia gravis* prompted a survey of serum from additional patients in this category. Our interest in such patients was made known to some 9000 physicians who had previously said that they were willing to co-operate in the research programmes of the National Institutes of Health's Clinical Center. Notification of the investigation also appeared in several medical periodicals. We have received to date, from physicians in many parts of the United States and Canada, serum samples, clinical summaries and histopathological slides of resected or biopsied tumours from 51 patients in this category. Similar material has been provided from 13 additional patients with thymomas *associated with myasthenia gravis*.

Clinical Data: Only patients with histopathologically documented thymomas, whose medical histories satisfactorily ruled out the presence of clinically apparent myasthenia gravis, were included in this study. The strenuous occupations and activities of several of these patients made it unlikely that they experienced abnormal or incapacitating muscular fatigue with exertion. Other clinical features of several patients in this group are considered below.

Histopathology: Sections of resected or biopsied tumours as well as histopathological reports were available on most patients in the series. Thymic tumours of lympho-epitheliomatous, spindle cell and granulomatous or Hodgkins' type are represented in the series of 51 non-myasthenic patients with thymomas. An independent coded review of this material by pathologists experienced in the classification of thymic tumours is in progress. Their findings and correlative serological data will be reported.

Methods: Serum specimens prepared from freshly drawn blood samples were sent to us by special delivery mail, in clean snap-cap vials, packed in tubular cardboard and metal containers at ambient temperatures. Aliquots for immediate study were stored at 4°C and additional aliquots for possible future studies were stored at −70°C.

The sera were examined by the indirect immunofluorescence technique for detection of γ-globulin binding as described in our previous specificity studies (Strauss *et al.*, 1965). In brief, composite cryostat sections of bovine skeletal muscle and calf thymus were sequentially treated with appropriate dilutions of test sera and ultraspecific fluorescein isothiocyanate-conjugated rabbit antibody to human γG-immunoglobulins. As we

TABLE II

THYMOMA STUDY: COMPARISON OF TITRES FOR SERUM γ-GLOBULIN REACTIONS WITH BOTH SKELETAL MUSCLE AND THYMUS†

Titre	*Myasthenia gravis without documented thymomas (number of patients)*	*Myasthenia gravis with thymomas (number of patients)*	*Thymoma without myasthenia gravis‡ (number of patients)*
1:60	19		
1:120	10	2	2
1:240	8	3	1
1:480	14	4	3
1:960	10	9	2
1:1920 (or more)	19	13	5
Totals of patients	80	31§	13

† This table presents combined data from the earlier study (Strauss *et al.*, 1965) and the present one.

‡ Where two or more titres were available from the same patient, the highest is tabulated

§ Two additional patients with myasthenia gravis and thymomas had unreactive serum.

have reported previously, the range of titres for reactive sera of patients with myasthenia gravis in our test system, was from 1:60 to 1:1920 (or greater). The same limits were determined for the present studies which were carried out between August 1964 and January 1965 together with the appropriate interposition of positive and negative controls. Serum samples were each retested at least three times during the period of study and no important variations in results were encountered during the many retests.

Results: Serum from 12 of the 51 patients with thymomas or histories thereof *without associated myasthenia gravis* reacted against both skeletal muscle striations and thymic epithelial cell cytoplasm. Titres were in the same range as had been observed with reactive

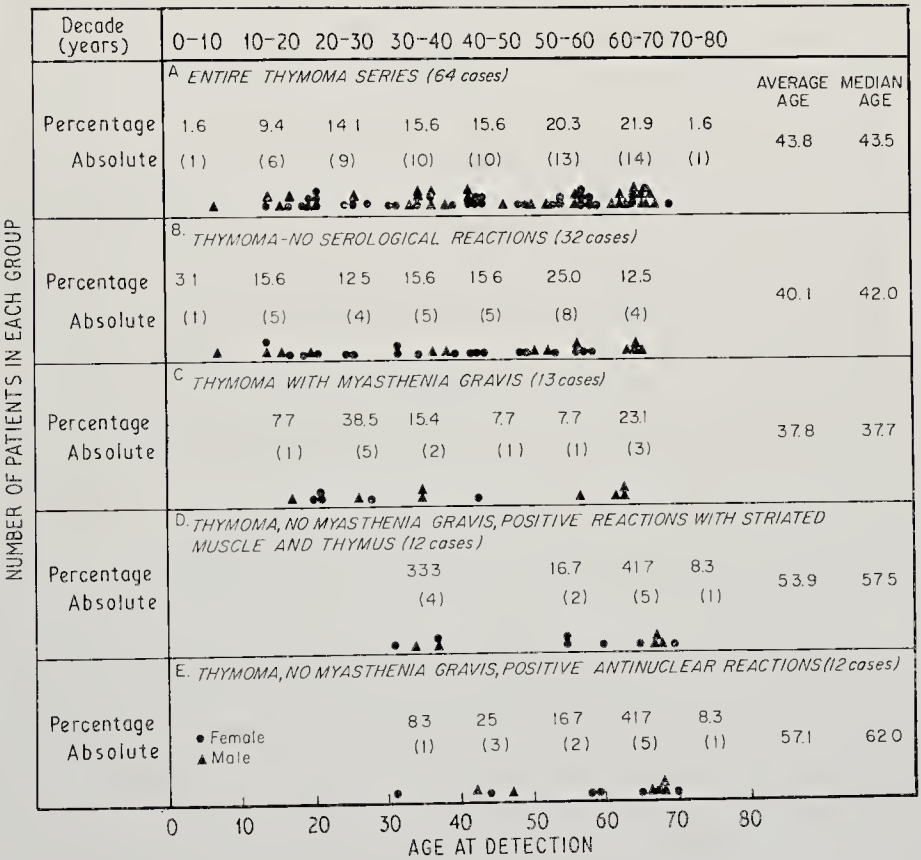


FIG. 2. Scatter diagrams illustrating distribution of ages of detection of thymomas in the present series of 64 patients, according to sex and other subgroups. Percentages: proportion of cases in the total group or in subgroups during each decade. Numbers in parenthesis: absolute numbers of cases in each decade.

sera from patients with myasthenia gravis (Table II). The serum from five of these patients also possessed antinuclear reactivity. Seven out of the 39 patients whose serum failed to react with skeletal muscle striations and thymic epithelial cells had serum

which possessed antinuclear reactivity; (serum antinuclear reactivity in myasthenia gravis was first described by White and Marshall [1962]). Twelve out of 13 additional patients with thymomas or histories thereof *with associated myasthenia gravis* whose serum was submitted in connexion with this supplemental study, reacted concurrently with skeletal muscle striations and thymic epithelial cells. Table II summarizes the titration data of our previous (Strauss *et al.*, 1965) and present study. It provides a comparison of the three groups of patients whose serum was reactive: (a) patients with myasthenia gravis not associated with documented thymomas; (b) patients with thymomas or histories thereof and associated myasthenia gravis; and (c) patients with thymomas or histories thereof unassociated with myasthenia gravis.

Comparison of the scatter diagrams (Fig. 2) for the distribution of the ages at which thymomas were first detected in the recently assembled group of 64 cases revealed the following additional features:

(a) In the group of patients *with* myasthenia gravis and reactive serum and in the group *without* myasthenia but with serum which also reacted with skeletal muscle and thymus, there were bimodal distributions of the ages at which thymomas were detected. In these two categories thymomas were most commonly diagnosed in the one or two decades before the age of forty and in the two decades after the age of fifty. In contrast, ages for detection of thymomas in patients with neither myasthenia gravis nor serological reactions with skeletal muscle and thymus, were relatively evenly distributed over the seven consecutive decades of life.

(b) Myasthenia gravis patients with thymomas were predominantly under forty years of age (average 37·8 years) at detection. In contrast, the thymoma patients with the same serological reactivities who did not have myasthenia

gravis were predominantly over fifty (average 53·9 years). The differences between the age distributions of the two groups were tested by the Wilcoxon Test (Wilcoxon, 1945) and were found to be significantly different ($P < 0{\cdot}05$).

(c) The thymoma patients with myasthenia gravis over the age of fifty were all males. The comparable age group among patients with thymomas *unassociated* with myasthenia gravis and with the same serological reactivities contained a slight preponderance of females.

Six of the twelve patients with thymomas or histories thereof *without clinically apparent myasthenia gravis* and whose serum reacted with skeletal muscle striations and thymic epithelial cell cytoplasm, were admitted to the National Institutes of Health's Clinical Center at Bethesda. The patients were studied by the staff of the Medical Neurology Branch, National Institutes of Neurological Diseases and Blindness and co-operating units. Each patient was given a thorough general physical and neurological examination designed to elicit evidence of weakness or abnormal fatigability of voluntary muscles. Every patient was examined electromyographically. No clinical evidence of myasthenia gravis could be demonstrated in any of these patients. It seemed reasonable to determine whether curare provocation tests could elicit latent myasthenia in the otherwise asymptomatic individuals who characterized this group: three such tests have been done. A 37-year-old Negro female, a housewife, whose thymoma had been resected in June 1964, was given appropriate placebos and five one-eightieths of the normal human curarizing dose of d-tubocurarine successively, intravenously, followed by an additional intravenous injection of one-twentieth of the normal curarizing dose. An anaesthetist was present throughout this procedure. Hand ergograms, which were recorded before the test, after the administration of placebo and after the injections of d-tubocurarine, were identical and normal. No other signs of curare-induced paresis were observed. The test was judged to be

unequivocally negative. Immediately before and two weeks after thymectomy the serum titres for muscle-binding and thymus-binding reactivities in this patient had been 1:240. On the day that the curare provocation test was performed, in mid-December 1964, the serum titres were greater than 1:1920.

A curare provocation test was also carried out on patient W.B. a 74-year-old Negro male, a retired postman, with an anterior mediastinal mass first detected, radiologically, seven years before our study. The titre of reactivity against skeletal muscle and thymus in this patient immediately after thymectomy was 1:480. Six months before our study he had been shown to have an aregenerative anaemia. The curare provocation test was conducted immediately before the induction of anaesthesia for resection of the patient's mediastinal tumour, and the result was negative. The resected anterior mediastinal tumour had the gross and histopathological features of a thymoma.

The third patient on whom a curare provocation test was performed was a 69-year-old white male agricultural labourer who had had a thymoma resected in 1964. His serum titre for anti-muscle and anti-thymus reactivities was 1:960 and for antinuclear reactivity 1:120 when measured several days before the curare provocation test. The result of the test in this instance was also negative.

Drs. Dale McFarlin and Mahlon Barlow of the National Institute of Neurological Diseases and Blindness and Dr. Gary Cage of the National Cancer Institute participated in the evaluation of these patients. A more extensive and detailed clinical report of their findings will be published later.

IMPLICATIONS OF THE PRESUMED AUTO-IMMUNE ASSOCIATIONS OF MYASTHENIA GRAVIS AND THEIR RELEVANCE TO THYMIC PATHOLOGY

The recent demonstration of concurrent immunoglobulin reactivities for skeletal muscle and thymus in the serum of

patients with thymomas *unassociated* with myasthenia gravis appears to indicate dramatically that the symptom-complex of myasthenia gravis is not an obligatory consequence of the presence of these antibodies in the circulation. The range of intervals between the initial detection of thymomas in this group of patients and our subsequent study of their sera (one month to thirteen years) suggests that antibodies to muscle and thymus can be present for long periods without the development of myasthenia gravis. It has been reported in two cases, however, that the symptoms of myasthenia gravis have emerged ten years after the initial detection of thymomas (Castleman, 1955; Klein *et al.*, 1964). Typically, though, thymoma and myasthenia gravis become manifest in the same patient within weeks or months of each other. The patients in our present study will be followed to detect the late development of myasthenia gravis. It is important also to find out if an extended series will sustain the observation that thymoma patients *with* myasthenia gravis and serological reactivities constitute a significantly younger group than thymoma patients with the same serological reactivities *unassociated* with myasthenia gravis. The explanation of this finding may be that patients with myasthenia gravis are likely to have earlier and more extensive X-ray studies than the other group. It may, however, indicate more fundamental differences between the two groups.

The biological significance of γ-globulins which react with both striated muscle and thymic epithelial cells in the sera of patients with myasthenia gravis is not yet determined. The high degree of disease specificity of this phenomenon suggests that it is intimately, though not necessarily causally, related to the processes whose ultimate clinical manifestations are the symptoms and pharmacological responses of myasthenia gravis. It is probably also significant that antibody reactivity in serum is directed against the two tissues which are characteristically involved in this disorder—skeletal muscle and thymus. Van der Geld, Feltkamp and Oosterhuis (1964) have produced evidence

from reciprocal absorption studies which suggests that the concurrent reactivities are related to common antigenic determinants in these two tissues. We have reported (Strauss *et al.*, 1966) that we have not yet been able to demonstrate any additional cross-reactive tissue sites with the exception of alternate striations in cardiac muscle; reactivity with the latter site was first described by Beutner and co-workers (1962).

Osserman and Weiner (1965) have confirmed an earlier *a priori* suggestion (Strauss *et al.*, 1960) that presumed auto-immune reactivity is most frequently demonstrated in the serum of patients with severe generalized myasthenia gravis of relatively short duration who also have demonstrable associated thymic pathology. On the other hand, the pharmacological response to anticholinesterase drugs and the electromyographic observations place the defect accounting for the symptoms and signs of the disease within the synaptic rather than in the contractile region of skeletal muscle. Investigators who are involved with the neurophysiology and pharmacology of myasthenia gravis might reasonably expect that, if the concept of auto-immunity is to be invoked to explain the pathogenesis of this disorder, antibodies with a specific affinity for sites in the synaptic region should be demonstrable. McFarlin, Engel and Strauss (1966), in an extensive study reported at the 1965 Conference on Myasthenia Gravis sponsored by the New York Academy of Sciences, were, however, unable to demonstrate by enzymatic staining in skeletal muscle a positive correlation between possible loci of immunofluorescence and structures identified as synaptic regions. These findings, although they do not absolutely preclude the existence of antibodies with an affinity for or an effect on such sites, indicate that such antibodies cannot be demonstrated by means which can, with relative ease, demonstrate reactions with striations, thymic epithelial cells, cell nuclei and so on.

Van der Geld, Feltkamp and Oosterhuis (1964) had reported the consistent occurrence of anti-muscle and anti-thymus reactivities

in nine patients with myasthenia gravis and associated thymomas. This finding, with few exceptions, has been confirmed by additional studies previously (Strauss *et al.*, 1965) and presently described. The fact that such antibodies are also present in the serum of patients with thymomas or histories thereof who do not have myasthenia gravis suggests that these antibodies are intimately related to pathology in the thymus. Several patients in our recent series with thymomas show, as well as antibodies in their serum, one or more of the other impairments known to occur with this tumour. These include hypogammaglobulinaemia, erythroid aplasia and leucopenia. Patient W.B. (this volume, p. 430), for instance, had a titre of reactivity against skeletal muscle striation and thymus of 1:480. Anaemia with erythroid aplasia in the bone marrow was demonstrated in him about six months before his thymectomy. Subsequent studies revealed leucopenia, a relative and absolute lymphopenia and hypogammaglobulinaemia with a normal turnover of exogenous radio-isotope-labelled γ-globulins. First set Caucasian skin homografts showed a prolonged survival (still surviving after 75 days). The patient also demonstrated an inability to become sensitized to 2,4-dinitrochlorobenzene in carefully controlled studies. When his peripheral blood leucocytes were cultured *in vitro* with phytohaemagglutinin the development of lymphoblasts, and mitogenesis, could not be elicited. Several other individuals in our series have shown combinations of various abnormalities including thymoma, erythroid aplasia, myasthenia gravis, migratory polyarthritis, antibodies to muscle and thymus, the presence of the antinuclear factor and hypogammaglobulinaemia (Strauss *et al.*, 1966). It is possible that a core process involving the thymus may, to a variable degree, produce other phenomena either singly or in combination. On the other hand, any one of these conditions may occur in the absence of demonstrable thymic pathology. This suggests that the thymus may be only one of several target organs which can be affected by a primary patho-

genic process. The mechanism by which myasthenia gravis itself is produced as the result of such a process remains to be elucidated. We would like to suggest a novel and speculative hypothesis which may help to connect two lines of thought and observation on this subject—the occurrence of specific immune serological reactions in patients with myasthenia gravis and thymic dysfunction and pathology, and the earlier concepts about the presence of presumed aetiological circulating inhibitors of neuromuscular transmission (reviewed by Nastuk, Strauss and Osserman, 1959); auto-antibodies are viewed in this hypothesis as indicators rather than causes of disease. Our hypothesis has been formulated as follows:

(a) It appears unlikely that auto-antibodies to muscle and thymus directly cause the primary disease changes which result in myasthenia gravis because (i) these antibodies do not apparently occur in the majority of patients with the disease even at a single point in time (Osserman and Weiner, 1965), (ii) these antibodies occur in some patients with thymomas *unassociated with myasthenia gravis* as we have shown in this paper, and (iii) these antibodies do not appear to react at sites corresponding to the assumed neuromuscular defect.

(b) Antibodies to muscle and thymus are almost invariably found in patients with thymomas and myasthenia gravis. Thymoma appears to represent a relatively late stage in a process of thymic pathology, the earlier manifestations of which are hyperplasia and germinal-follicle formation. Good and Papermaster (1964), in a masterly review on the ontogeny and phylogeny of adaptive immunity, have compiled data from many sources to support the thesis that the development of specific immunity is a unique feature of vertebrate evolution. These workers have shown that the emergence of specific immunity closely parallels the development of a lymphoid thymus, other organized lymphoid tissues and the lymphoid-cell system. They further suggest that lymphoid cells and the thymus, and organs intimately

associated with the development and control of these, may have evolved not exclusively as a protecting mechanism against exogenous infectious agents, but also to cope with antigenic changes (at cell surfaces) in cells of the complex organ-systems of vertebrates. "Such surface changes may herald the formation of cell lines no longer susceptible to aggregational and other ordering forces associated with the differentiation and maintenance of normal organ structure." (Good and Papermaster, 1964, p. 94.) We have suggested elsewhere that antibodies directed against thymic epithelial cell cytoplasm (and this includes cell membranes) may develop in response to an antigenic change which presages the development of thymic neoplasia (van der Geld, Feltkamp and Oosterhuis, 1964; Strauss *et al.*, 1965). The generally non-metastatic character of thymomas may relate to successful immunological cytolysis of cells which break away from the main tumour mass. It is conceivable that the archetype of the "policing" role of the lymphoid system suggested by Good and Papermaster (1964) may indeed be represented in the immune response to the thymic lympho-epithelioma. Such a self-reflexive mechanism suggests to us the illusion of: "A watchbird watching a watchbird."

(c) The concept of a circulating neurohumoral inhibitor has been advanced for the pathogenesis of myasthenia gravis, because transient neonatal myasthenia gravis has been observed in infants born to mothers with the disease. It is possible that the thymus, in the course of its derangement, elaborates such a substance and this may even be a subunit of a normal constituent of the thymic epithelial cell. Such a substance might be small enough to enter into intact muscle fibres and, because of analogous configuration to sites within the A-band, to interfere with normal metabolic processes in this region. Such a reaction might result in a retrograde effect which would be interpreted primarily as a defect in neuromuscular transmission. The same substance might also, as a physiological defence against neoplasia or as a consequence of the

abandonment of self recognition, function as an antigen. Failure to demonstrate antibodies to this hypothetical substance in the majority of patients with myasthenia gravis might be due either to the maintenance of unimpaired self-recognition in such patients or to an irregular physiological immune response to neoplasia. The presence of antibodies to muscle A-bands and thymus might thus be a reflection of the factors involved in the pathogenesis of myasthenia gravis. It could be, however, that such antibodies function defensively to inactivate the proposed antigenic neuro-humoral inhibitor. The significantly increased presence of such antibodies in patients with myasthenia gravis of recent onset and marked severity might reflect the presence of increasing quantities of such an inhibitor which is, at the same time, antigenic. The presence of such antibodies in persons with thymomas unassociated with myasthenia gravis might, on the other hand, represent the successful inactivation of such a substance.

This hypothesis, while highly speculative, is not beyond ultimate experimental verification, modification or refutation. At least it has the virtue of accounting for many of the recognized fragments of data concerning the pathogenesis of myasthenia gravis, its relation to auto-immunity and thymic pathology.

SUMMARY

Pathology in the thymus in man has been observed in association with a number of clinical disorders in which immunological derangements or serological auto-immune concomitants are present. Recent experimental findings indicate that the thymus plays an important role in the development of immunocompetency. These findings suggest interesting lines of investigation for the elucidation of possible functional relationships between thymic abnormalities and disease states.

Our investigations have dealt with concurrent immune reactivity against striated muscle and thymic epithelial cells, first

observed *in vitro* with serum from patients with myasthenia gravis. This reactivity, tentatively classed as auto-immune, is most consistently demonstrable with serum from patients with myasthenia gravis who also have thymomas. It is rarely observed with serum from normal and disease-control individuals. More recently, the same reactivity has been observed in a similar range of titres in the serum from 12 of 51 patients with thymomas who *did not* have myasthenia gravis. It seems, therefore, that myasthenia gravis is not an obligatory consequence of the presence of such reactivity in serum but that this reactivity is most intimately related to the presence of thymomas.

A hypothesis has been developed to connect the observation of specific immune serological reactions in patients with myasthenia gravis and/or thymomas not associated with myasthenia gravis, and our present views on thymic function and pathology, with earlier concepts that circulating inhibitors of neuromuscular transmission are involved in the pathogenesis of myasthenia gravis. Auto-antibodies to skeletal muscle and thymus are viewed as indicators with possible protective activity rather than as causes of disease. It is suggested that the immune response to a thymoma may constitute the archetype manifestation of the endogenous "policing" function of the lymphoid system as postulated by Good and Papermaster (1964).

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DISCUSSION

Burnet: Dr. Strauss, can you tell us anything about the changes in the serological reactions that are associated with thymectomy in myasthenic patients?

Strauss: Various workers have studied this matter independently (Osserman, K. E., and Weiner, L. B. [1965]. *Ann. N.Y. Acad. Sci.*, **124**, 730; [1965]. *New Engl. J. Med.*, **273**, 615; Nastuk, W. L., personal communication; Van der Geld, H. W. R., personal communication). They are all of the impression—but this can, in my view, only be an impression—that after thymectomy the serological reactivity slowly decreases and may eventually disappear altogether. I do not believe that we yet have enough base-line data about the normal fluctuations of this reactivity, with time and without surgical intervention, to say anything more definite about it than that.

Burnet: E. H. Beutner in Buffalo (personal communication) has reported to me that these reactions are usually much stronger in patients

with thymomas than in patients without them. Do you agree with this?

Strauss: Serological reactivity is almost universally present in patients with myasthenia gravis and associated thymomas, and I agree that the thymoma patients with myasthenia are generally at the upper end of the scale of serum reactivity, but there is considerable overlap. For example, we have seen very high titres in myasthenics in whom the thymus is totally absent at post mortem. We have conjectured that the thymus may have been eliminated by immunological means in these cases. This picture of absent thymus and a high titre of serum reactivity usually occurs in male patients with florid myasthenia in old age.

Good: The relationship of myasthenia gravis to auto-immune and immunological phenomena in the thymus is puzzling as Dr. Strauss has commented in his presentation. Drs. M. Blaw, R. D. A. Peterson and I (unpublished material) have removed the thymus of newborn C3H and Swiss mice and then tested these animals for sensitivity to curare. We found, surprisingly, that the neonatally thymectomized and not the sham-operated animals showed a twofold to fourfold increase in their sensitivity to curare. We are now studying the serum of myasthenics to see if it contains an active, curare-like substance. Perhaps this could be a mechanism for detoxifying naturally occurring toxins that may be present in the lymphoid system.

Mackay: It is perplexing that the obvious auto-antibody in myasthenia gravis is not one that would fit with the neurophysiology of the disease. Children of myasthenic mothers may have transient myasthenia at birth, yet I understand from Dr. Strauss that certain of these mothers have been tested and do not have the auto-antibody in their serum. There may, perhaps, be a parallel here with a disease like pernicious anaemia in which there is more than one auto-antibody to gastric components. One of these auto-antibodies, easy to demonstrate, is to the parietal cells of the stomach, whilst the other, to intrinsic factor, can be demonstrated only with great difficulty and by a complex assay technique. Perhaps there is also more than one circulating auto-antibody in myasthenia gravis.

It is noteworthy that various muscle-destroying diseases listed by Strauss are not associated with auto-antibody to muscle, since there is a

school of thought which believes that auto-antibody occurs merely as a response to tissue breakdown products. If we can exclude this possibility, any explanation of myasthenia gravis must account for the specific myasthenia disease syndrome, the presence of the auto-antibody to muscle and, in some cases, discrete lymphocytic foci in muscle. The application of Ockham's razor gives us fairly good grounds for calling myasthenia gravis an auto-immune disease.

Metcalf: Dr. Strauss, am I right in interpreting from your slides that the thymus sections invariably show some true epithelial cells that do *not* fluoresce? If so, this would be a fine discriminatory tool and it would also confirm our ideas of heterogeneity in the epithelial-cell system.

Strauss: This is not a general phenomenon, but in about half a dozen thymomas from patients with myasthenia gravis we have found that only the minority of thymic epithelial cells react. With material from human foetuses and from calves all the epithelial cells, or at least all the cells commonly thought of as such, react.

Metcalf: But your photomicrographs showed what looked like islands of positively reacting cells in which there were isolated epithelial cells that were non-reactive.

Strauss: We most frequently encounter these cells in clusters of three to fifteen, predominantly in the medullary zones. We see occasional single cells in section, which may be cortical or medullary, and which also react with sera from patients with myasthenia. When the sections are restained with haematoxylin and eosin all the reactive cells have the staining properties usually associated with thymic epithelial cells. We are at the moment trying to find out if all these cells are derived from embryonic epithelium.

Stanley: Professor Dameshek, you have described the progression of disease in one direction—from auto-immunity to neoplasia. How common, clinically and experimentally, is the reverse situation in which patients with neoplasia or leukaemia later show auto-immune phenomena?

Dameshek: Very common. In my presentation I intended emphasizing the auto-immune reaction which progresses to leukaemia but, from the clinical standpoint, the reverse situation is more common. In the chronic lymphoproliferative disorders such as lymphocytic leukaemia,

auto-immune reactions may occur early or late—and frequently very late—in the disease. It is in these very late stages that there are often a host of immunological abnormalities.

Stanley: Is it possible that the aetiological agent in these diseases is a virus which transforms cells so that they react against the host in two ways, either by producing malignant changes or by causing immunological injury?

Dameshek: I did not want to discuss viruses, or the thymus, in my presentation because Professor Good knows much more about the thymus than I do and, as for viruses, I know nothing about them at all! It is certainly possible that a virus may initiate the abnormal clone. I do not think we have necessarily to postulate that everything that comes after this initiation has to be due to the virus.

Stanley: I agree.

Rowley: Dr. Strauss, your test can only have a causal interpretation if it can be shown that the fluorescence is due to an antibody to muscle. I understand that your colleagues have tried, but have not yet been able, to demonstrate such an antigen-antibody system. Is this correct?

Strauss: I would like to clarify some of the points about the techniques we use as there seems to be some misunderstanding about them. The demonstration of antibody to fixed guinea-pig complement in the procedure we described (Strauss, A. J. L., *et al.* [1960]. *Proc. Soc. exp. Biol. Med.*, **105**, 104) required first the binding of guinea-pig complement, and antibody could not be demonstrated until this had been done. Appropriate controls were negative so there must be something in myasthenic serum which causes complement fixation *in vitro*. Three different types of immunofluorescent staining have been used by us, and others, for the study of reactivity of myasthenic serum to striated muscle and thymus *in vitro:* the direct, the complement staining, and the indirect. Findings with all three methods support the thesis that quantitatively and qualitatively unique factors which react in striated muscle and thymus are present in serum from patients with myasthenia gravis. In answer to Professor Rowley's questions it should be stressed that the binding of this factor or factors is not dependent on fixation of guinea-pig complement—complement staining is just an indicator system. We tend to think that the reactivity is caused by antibody in the patients' serum because it is the immunoglobulin fraction that is

reactive and this does, at least in part, fix complement when bound to our tissue substrates. Other explanations for this phenomenon may come to light with time and we should, of course, be prepared to consider these. We are nevertheless dealing with a highly disease-specific phenomenon within our test limits.

We initially used the direct technique in which a sodium-sulphate-precipitated globulin was conjugated with fluorescein isothiocyanate (Strauss, *et al.* [1960]. *Proc. Soc. exp. Biol. Med.*, **105**, 184). Later, we employed the so-called complement-staining technique first described by P. Klein and P. Burkholder (1959. *Dt. med. Wschr.*, **84**, 2001). In this procedure for the immunohistological demonstration of complement fixation, sections were treated sequentially with unconjugated serum from patients, guinea-pig complement and a fluorescent antibody directed against guinea-pig complement; appropriate controls were interposed which were negative. The third procedure—the indirect technique which we have employed in our present study—was first used in this field by Beutner (Beutner, E. H., *et al.* [1962]. *J. Am. med. Ass.*, **182**, 46). Sections were treated sequentially with patients' sera in various dilutions and then with an ultraspecific fluorescein-conjugated, rabbit antihuman immunoglobulin reagent. From these three procedures we have some very clear information about the nature of the reactant in the serum. Short of understanding the actual mechanism which gives rise to the serological reactivity, and the precise character of the reactants in striated muscle and thymus we can at least say there is circumstantial evidence that this material in the serum of patients with myasthenia gravis behaves like an antibody.

Irvine: We have heard that thymectomy does not have much effect on the auto-immune process in mice. In patients with myasthenia gravis without thymomas there is sometimes an improvement in their condition after thymectomy, but this may be only temporary. Dr. Strauss, what are your views on the place of thymectomy in the management of adult patients with other diseases associated with auto-immunity, for instance, systemic lupus erythematosus?

Strauss: It seems that thymectomy, if anything, actually accelerates the auto-immune process in mice. In myasthenia gravis, as you say, some favourable results have followed thymectomy and sometimes remissions after this operation have been permanent and striking.

Various clinical studies of thymectomy in myasthenia have suggested that the results of thymectomy may be better than the results of conservative pharmacological management in selected groups of patients (Schwab, R. S., and Leland, C. C. [1953]. *J. Am. med. Ass.*, **153**, 1270; Keynes, G. [1954]. *Lancet*, **1**, 1197; Eaton, L. M., and Clagett, O. T. [1955]. *Am. J. Med.*, **19**, 703; Simpson, J. A. [1958]. *Brain*, **81**, 111; Genkins, G., *et al.*, [1960]. *Bull. N.Y. Acad. Med.*, **36**, 826). The most favourable results after thymectomy are probably in young female patients with myasthenia of short duration and no thymic tumours. In one series sex did not seem to be a major factor (Genkins *et al.*, *loc. cit.*). No prospective matched group-studies have been done yet on the effect of thymectomy in this disease, and double-blind trials are not possible because there is no equivalent of sham-thymectomy in human patients for obvious medico-legal reasons. I could not predict what the value of thymectomy in systemic lupus might be. If this syndrome is anything like haemolytic disease in NZB mice thymectomy would be quite undesirable.

Metcalf: R. A. Joske (1958. *Med. J. Aust.*, **2**, 859) showed that patients with myasthenia gravis developed a relative lymphocytopenia after thymectomy. Professor Dameshek, do you know if any work has been done on this recently? If patients are followed for long enough after thymectomy do they develop significant immunological deficiencies as do mice thymectomized in adult life?

Dameshek: We followed a large group of myasthenics at the Massachusetts General Hospital to trace the effect of thymectomy on their blood and on the late occurrence of immunological abnormalities (Adner, M. M., *et al.* [1964]. *New Engl. J. Med.*, **271**, 1327). Lymphocytopenia, sometimes quite marked, developed in most cases and the more striking this was, the more likely it was that a disturbance of delayed hypersensitivity would be found. We also found occasional instances of systemic lupus and thyroiditis, and rheumatoid factor and antinuclear factor were seen a little more often than in a matched group of controls. But there was no significant evidence of an increased incidence of auto-immune disease in these cases.

Kaplan: R. S. Schwartz and L. Beldotti studied the production of F_1-hybrid wasting disease (1965. *Science*, **149**, 1151). They observed an increase in lymphoid neoplasms in the long-term survivors with this

disease. Dr. Schwartz injected C57BL cells into (C57BL × DBA) F_1 hybrid mice. Both the C57BL and the DBA strains have a high spontaneous incidence of precisely this type of reticulum-cell neoplasm in old age, that is, after they are twelve months old, and, in general, all F_1 hybrids of C57BL parentage exhibit this neoplasm with advancing age. I think that Dr. Schwartz' control mice did not complete their normal life-span but were killed at about twelve months so we should accept Schwartz' conclusions about the *induction* of lymphoid tumours in his hybrid mice with caution. He may simply have observed an *acceleration* of a natural process which we know from the classical descriptions of Thelma B. Dunn (1954. *J. natn. Cancer Inst.*, **14**, 1281) has a high spontaneous incidence in old mice.

I would also like to comment on the word "leukaemia". Those of us who have worked in this area for a long time are very sensitive to the use of this word. The thymic lymphomas, for instance, represent a completely different class of lesion from other lymphoid neoplasms and if we class all these diseases together as "leukaemia" there will be great confusion. Dunn used the term "reticulum-cell sarcoma" for the class of lesion observed by Schwartz. It will avoid confusion if we continue to use this term to distinguish between this neoplasm and the kind of lymphoma I discussed in my presentation. The reticulum-cell sarcomas develop in about 60–80 per cent of old, hybrid mice.

Furth: I, too, am interested in the problem of whether or not runt disease can terminate in leukaemia. We agree with Dr. Kaplan that neoplasms of lymphopoietic tissues are extremely common in ageing mice and that they do not originate in the thymus. Dunn's classification (1954, *loc. cit.*) of these diseases is purely morphological: she calls them neoplasms of reticular tissue. Some of them are lymphomas, leukaemic or aleukaemic, others are, morphologically, neoplasms of reticulum cells and some resemble granulomas. We have tried to transplant some of these "reticulum-cell tumours" into syngeneic hosts without success. A precise characterization of "reticulum-cell" sarcomas and the differentiation of leukaemia from leukaemoid lesions are yet to be worked out.

Dameshek: I attempted to define leukaemia some years ago and found it a difficult task. (Dameshek, W., and Gunz, F. W. [1964]. *In* Leukemia, p. 12, 2nd edn. New York: Grune and Stratton). A neoplastic,

generalized, self-perpetuating disturbance of one of the white-cell tissues might cover it, I suppose. But these generalized neoplasms that I have called leukaemia are transplantable and highly invasive. Some of Dr. Kaplan's comments are rather disconcerting and surprising to me. I have discussed these points with Dr. Schwartz and it would seem that since mice of all strains develop neoplasia, *any* experiment leading to a high incidence of malignancy in this species could be interpreted as accelerating the appearance of tumours rather than inducing their development. In view of our present ignorance of the neoplastic process, the argument of acceleration or induction becomes circular and cannot probably be solved until more data are available. C57BL mice are generally considered to be a low-leukaemic strain (Kaplan, H. S. [1948]. *J. natn. Cancer Inst.*, **8**, 191). The incidence of leukaemia in the subline used in Schwartz' experiments, C57BL/6J, was found to be approximately 1 per cent. This compares favourably with the incidence of 1·3 per cent found by M. Lieberman and H. S. Kaplan (1959. *Science*, **130**, 387) in their subline, C57BL/Ka. Some C57BL mice developed a reticulum-cell sarcoma classified by Dunn (1954, *loc. cit.*) as Type A. This type of tumour has not been observed in either the control or the experimental groups in Schwartz' study.

The incidence of leukaemia in C57BL/6J mice (donor strain) may not be relevant because none of the tumours that developed in the $(C57BL/6J \times DBA/2)F_1$ recipients behaved like donor-strain tissue. These tumours could not be transplanted to C57BL/6J mice; in contrast, the tumours were rapidly and uniformly fatal in syngeneic recipients (Schwartz, R., and Beldotti, L. [1965]. *Science*, **149**, 1511). These malignancies seem to arise, therefore, from host—$(C57BL/6J \times DBA/2)F_1$—cells in the course of the immunological graft-versus-host reaction.

Good: Professor Dameshek has told us that the immunoblast can give rise to both plasma cells and the lymphocyte series of cells. He also suggested that the immunoblast may be derived from dedifferentiation of the small lymphocyte. But is there any direct evidence that the cells which are derived from this small lymphocyte ever really enter the plasma-cell series? K. A. Porter and E. H. Cooper (1962. *J. exp. Med.*, **115**, 997) and J. L. Gowans (1962. *Ann. N.Y. Acad. Sci.*, **99**, 432) have described the dedifferentiation of cells into basophilic blast cells, but

do we know if these systems can produce any cell other than more small lymphocytes?

Gowans: No, Professor Good is quite right. There is as yet no direct evidence that plasma cells take origin from small lymphocytes, but the indirect evidence for this is very strong. We know that small lymphocytes can develop into dividing blast cells after antigenic stimulation, and we know that small lymphocytes can restore the ability to make antibody in immunologically deficient animals. But the direct demonstration is still lacking.

Woodruff: The effect of thymectomy on patients with myasthenia gravis really confuses the issue. Apart from this, are there any theoretical reasons for us to assume that thymectomy might affect an autoimmune process in a beneficial way? If it is true that the thymus is a place where primitive cells become immunocytes and learn in some way to recognize, and refrain from reacting against, other constituents of the individual's own body, and if an unwanted clone has somehow arisen, then I cannot see by what sort of mechanism thymectomy could be expected to deal with the situation. Sir Macfarlane, would you try to dispel my confusion by recapitulating the theoretical reasoning behind this?

Burnet: If a situation arises in which a clone, abnormally reactive against self-components, is multiplying in the body, then probably this clone can also break through the normal inhibition to multiplication in the thymus and multiply there as well. I have been impressed by reports of two infants with acquired haemolytic anaemia and a positive Coombs' test who were cured, after splenectomy and repeated blood transfusions had failed, by thymectomy. Both these babies are still alive and I have examined sections of the thymus from one of them. This thymus was full of plasmablasts and plasma cells and I am reasonably certain that large amounts of antibody, or at least of immunoglobulins, were being produced in it. This does give a rationale for thymectomy, at least in this particular case. If a pathogenic clone is multiplying rapidly in a young person in the thymus—and this is apparently a suitable place for the multiplication of such a clone—then the removal of a large fraction of the multiplying pathogenic cells with the thymus seems to be a rational procedure. On these general grounds I would say that thymectomy, even in myasthenia gravis, is only justifiable

in children and young adolescents. Dr. Mackay does not agree with me over this. In youth there is good evidence of cellular multiplication, presumably of pathogenic clones, in the thymus. In adults, the thymus is involuted and the proportion of these reactive and multiplying cells that could be removed by thymectomy is probably immaterial. My attitude is that if a very young person has a potentially lethal auto-immune disease it is worth considering thymectomy.

Mackay: Most thymectomies for mysasthenia gravis are performed on young adults. We have done thymectomies on three females, aged 14, 20 and 56 years, with systemic lupus erythematosus but even after three years we cannot really say whether thymectomy has improved their disease or not.

HISTOPATHOLOGY OF THE HUMAN THYMUS

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PHYSICIANS concerned with human disease owe a deep debt of gratitude to experimental scientists working in the field of thymus research over the past decade. The knowledge, accumulated by many of the contributors to this volume, has greatly helped our understanding of the *modus operandi* of the human thymus in health and disease—previously this was a closed book to the physician. It was this work of the experimental scientists and the encouragement of Sir Macfarlane Burnet that stimulated our present interest in the pathology of the human thymus.

The human thymus resembles that of other mammals in being a lympho-epithelial organ composed of a loosely cellular medulla and a compact lymphoid cortex. The architecture of the cortex and medulla is similar: there are the reticular-epithelial cells with interconnecting cytoplasmic extensions, arranged singly or in small clumps, surrounded by medium and small lymphocytes. Variable numbers of the reticular-eipthelial cells in the cortex and medulla stain intensely with periodic acid-Schiff (PAS). The Hassall's corpuscles of the medulla are complex tubular structures with features suggestive of a derivation from either epithelial cells or thick walled venules. The normal human thymus at all ages characteristically contains no germinal centres and few plasma cells. Pathological changes in the human thymus must be interpreted in relation to the marked parenchymal shrinkage that occurs with advancing age, when fatty tissue invades the thymic

lobules and the cortex tends to disappear. The shrunken thymus may contain small clusters or strands of spindle-shaped cells, and the pathological thymus may contain a range of cell types, some of which are assumed, on the basis of morphology and staining properties, to be derived from the epithelial cells. However, despite gross degrees of pathological change, the characteristic lympho-epithelial interrelationship tends to be preserved.

The main pathological changes in the human thymus will be described under the four headings of *aplasia, hyperplasia, dysplasia, and neoplasia.*

THYMIC APLASIA

Thymic aplasia may be *primary*, or *secondary* to "stress" such as malnutrition or severe illness. Secondary aplasia is mediated in part at least by cortisol.

Primary aplasia of the thymus is a rare disorder of infancy in which the thymus fails to undergo normal development in foetal life and is small and rudimentary from birth. This thymic aplasia appears to determine the lymphopenic subtype of agammaglobulinaemia; in the more usual types of agammaglobulinaemia due to plasma-cell deficiency, the thymus and lymphoid system are intact. In primary aplasia of the thymus, there is an extremely low level of lymphocytes in the blood, non-development of peripheral lymphoid tissues which lack germinal centres and plasma cells, agammaglobulinaemia, a deficiency in all types of immune function demonstrable by appropriate testing, a high susceptibility to infections, and early death (Rosen, Gitlin and Janeway, 1962; Peterson, Cooper and Good, 1965). This condition is of interest in being a clinical counterpart of the state associated with neonatal thymectomy in experimental animals: it is probably due to the combined effects of failure of thymic lymphocyte production and a deficiency of the lymphotrophic hormone of the thymus.

THYMIC HYPERPLASIA

In the 1910–1920 era there were many references by surgeons and pathologists to the association of thymic enlargement with thyrotoxicosis (Crotti, 1938), and thymectomy was even performed as a treatment for thyrotoxicosis. Thyrotoxicosis was amongst the diseases chosen by Hammar (1929) when he analysed

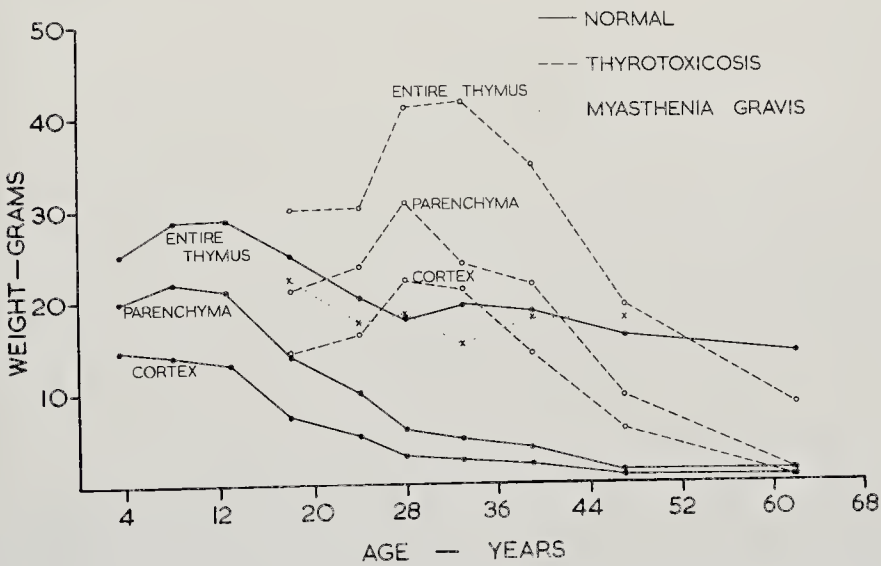

Fig. 1. The weight was greater for all components of the thymus in 43 cases of thyrotoxicosis than in "normal" control subjects (victims of sudden accidental death). Each point represents a mean value of weights for all cases within successive five-year periods of life. Data derived from Hammar (1929). A similarly derived graph, which does not differ from the normal, for myasthenia gravis was constructed from data of Castleman (1955), who cited the weight of the entire thymus in 143 cases of myasthenia gravis.

the weights of the thymus and its components. To obtain an age-controlled measure of the thymic weight in thyrotoxicosis, I arranged Hammar's 43 cases of thyrotoxicosis into five-year age groups corresponding to the five-year age periods into which Hammar placed his control cases, these being victims of sudden accidental death. The range of thymic weights for each age group was fairly wide, but the mean weights for all thymic

components were considerably greater in thyrotoxicosis than in the controls, particularly in the younger subjects (Fig. 1). The increased epithelial activity is reflected by the graph (Fig. 2) derived from Hammar (1929), showing the greatly increased number of Hassall's corpuscles in thyrotoxicosis.

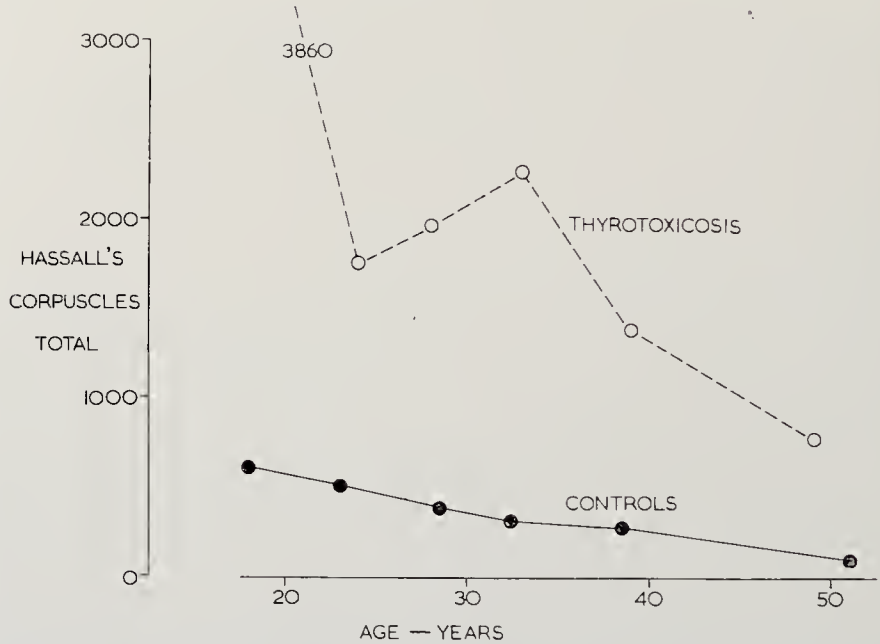

FIG. 2. Graph showing increased numbers of Hassall's corpuscles in thymic medulla in thyrotoxicosis compared with "normal" control subjects. Points on these graphs were derived, as in Fig. 1, from data of Hammar (1929).

The thymus was examined histologically in six of our cases of thyrotoxicosis, and in five there was evidence of hyperplasia of epithelial tissue in the medulla and increased thickness of the cortex. Germinal centres were present in one case—a female aged 29 years with coexisting thyroiditis. Gunn, Michie and Irvine (1964) obtained biopsies of the human thymus in the course of surgical thyroidectomy for thyrotoxicosis, and found germinal centres in the medulla in as many as 16 out of 50 thymic biopsies.

Relatively large amounts of thyroxine were shown by Warner (1964) to increase the thymic cortex in fowls, suggesting that thyroxine acted as a physiological stimulus, perhaps on the epithelial cells of the medulla. Thus, in thyrotoxicosis, thyroxine may be responsible for thymic enlargement which in turn is responsible for the accompanying peripheral lymphocytosis and lymphoid hyperplasia. However this explanation does not satisfactorily account for the germinal centre change, which points more to a participation of the thymus in an auto-immune reaction now believed to be one cause of thyrotoxicosis.

It is frequently stated that there is thymic hyperplasia in myasthenia gravis because germinal centres are present in the thymic medulla. However, thymic hyperplasia in the sense of increased tissue mass was usually not present in the series wherein Castleman (1955) cited the total weight of the non-thymomatous thymus in 143 cases of myasthenia gravis (Fig. 1).

THYMIC DYSPLASIA

The term dysplasia is used to denote changes in thymic structure as a result of disease, exemplified by the presence of lymph follicles in the medulla in myasthenia gravis and by epithelial aggregates in the medulla in lupus erythematosus.

In seven of our ten cases of myasthenia gravis, the medulla contained lymph follicles with germinal centres identical with those seen in a stimulated lymph node: this change in the thymus is interpreted as an immunological response to stimulation by an antigen. This antigen is almost certainly an intrinsic or auto-antigen, and is most probably a constituent of the thymus itself. Serological tests in myasthenia gravis show that in 30 per cent of cases there is an auto-antibody to the A-bands of skeletal muscle. Moreover, sera which react with muscle also react with a component of thymic epithelial cells (Strauss *et al.*, 1965), implying that the auto-antigen is common to muscle and to thymus

epithelium. If the thymic epithelial cells represented the main source of common antigenic determinant responsible for stimulating the forbidden clones concerned to proliferate, this would be a feasible explanation for the benefit derived from surgical thymectomy in myasthenia gravis.

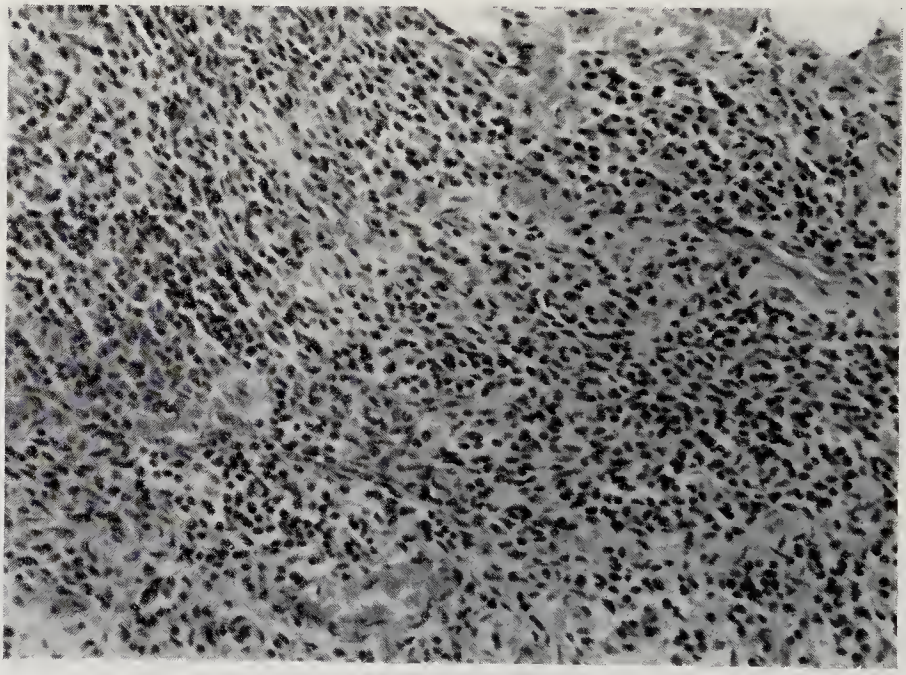

Fig. 3. Area of spindle-epithelial cells in thymic medulla of a female aged 14 years with lupus erythematosus. Case reported by Burnet and Mackay (1965). Haematoxylin and eosin. (× 270).

In our nine cases of systemic lupus erythematosus there were changes in the thymic medulla of a different character from those of myasthenia gravis. The thymus was small since the entire weight in five of our cases ranged between 5·0 and 9·6 grams. The histological changes included gross lymphoid depletion of the cortex and medulla, collapse of the normal architecture as shown by reticulin staining, and accumulations of cells in the medulla which, depending on the plane of the section, appeared as

either rounded "islands" or elongated "archipelagos" (Hutchins and Harvey, 1964). These cells occasionally resembled epithelial cells but were mostly spindle-shaped (Fig. 3); they often resembled the cell type which constitutes spindle-cell thymomas and the cells of thymic grafts which have survived in diffusion chambers.

Since small areas of spindle-epithelial cells may be present in any shrunken thymus (Castleman, 1955; Lattes, 1962), a quantitative assessment of spindle-epithelial cell areas and other histo-

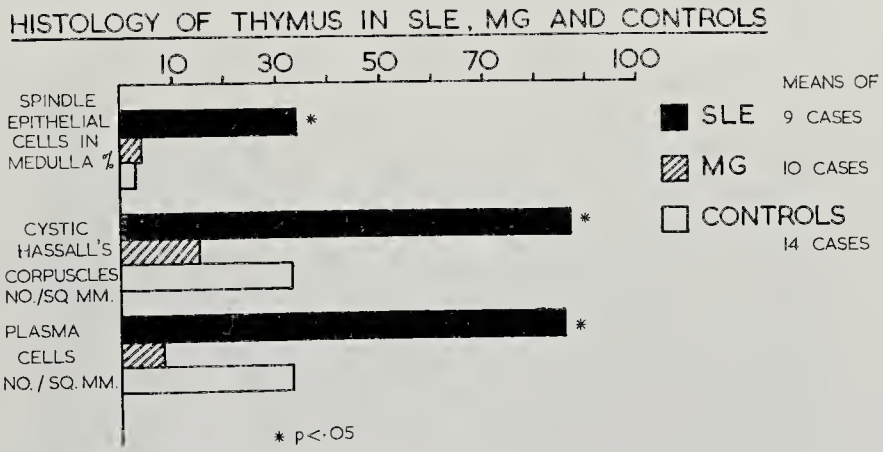

FIG. 4. Percentage areas of spindle-epithelial cells in medulla, and numbers of cystic Hassall's corpuscles and plasma cells per mm². of a thymic section. Mean values for 9 cases of systemic lupus erythematosus (SLE), 10 of myasthenia gravis (MG), and 14 age-matched controls with miscellaneous diseases. All values for SLE were significantly increased ($P < 0.05$).

logical features of the thymus was developed (Goldstein and Mackay, 1965). This involved the projection on to paper of standard areas of thymic sections, and weighing the outlines of the various thymic components to obtain their relative areas; the smaller structures in the standard area, including cells and Hassall's corpuscles, were counted. In nine cases of systemic lupus erythematosus, ten of myasthenia gravis and 14 age-matched controls, a mean value was obtained for the various components of the thymus.

In lupus erythematosus, as compared with the other two groups, there was a statistically significant increase in the proportion of the medulla occupied by spindle-epithelial cells, and also in the number of plasma cells and cystic Hassall's corpuscles (Fig. 4); there was a lesser increase in mast cells. The epithelial accumulations contained numbers of PAS-positive cells and

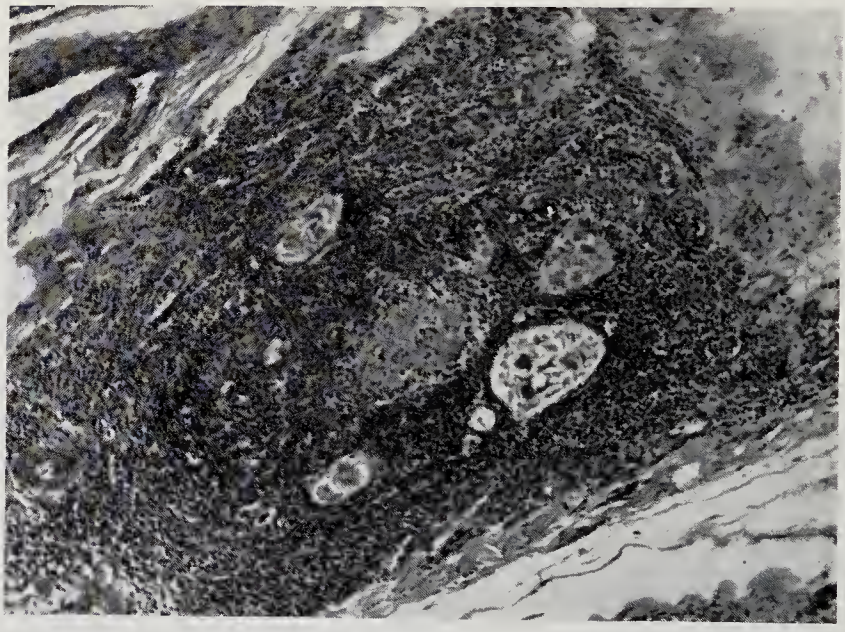

Fig. 5. Area of medulla of thymus from a female aged 17 years with lupus erythematosus. Case reported by Mackay, Masel and Burnet (1964). Note accumulation of spindle-epithelial cells around extracellular eosinophilic and PAS-positive material. Haematoxylin and eosin. (× 100).

extracellular eosinophilic PAS-positive material (Fig. 5); the relative numbers of the PAS-positive cells were not analysed. In two cases typical germinal centres were present in the medulla. In myasthenia gravis, the only significant change was germinal centre formation in the medulla.

There appeared to be a relatively increased area of spindle-epithelial cells in the medulla in certain other conditions (Fig. 6), including rheumatoid arthritis, the Goodpasture syndrome of

nephritis and pulmonary haemorrhage, chronic hepatitis, although only small numbers of cases have so far been studied, and also in the thymus of two patients with chronic glomerulonephritis who had both received a renal homotransplant.

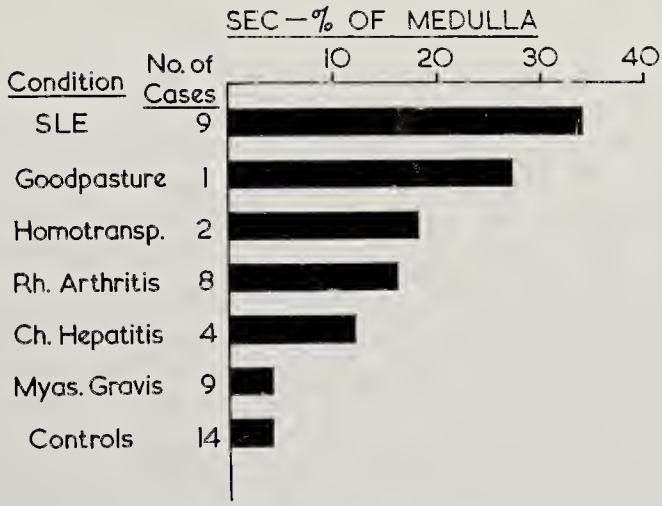

FIG. 6. Percentage area of spindle-epithelial cells (SEC) in medulla in systemic lupus erythematosus (SLE), Goodpasture syndrome, recipients of renal homotransplant, rheumatoid arthritis, chronic hepatitis, myasthenia gravis and controls with miscellaneous diseases. Mean values for stated number of cases.

Thymic sections which were made available to us from patients not included in the above analysis showed features of interest. A female patient aged 60 years had myasthenia gravis and positive tests for L.E. cells and antinuclear factor: there was cortical atrophy and the medulla contained areas of spindle-epithelial cells, as seen in sytemic lupus erythematosus, but no germinal centres. The surgically resected thymus from two infants with auto-immune haemolytic anaemia showed prominent areas of PAS-positive cells and in one case plasmablasts. Sections of a thymus from a child of 14 years who died acutely with the Goodpasture syndrome showed invasion of the cortex by epithelial

cell aggregates, a pronounced degree of epithelial proliferation in the medulla, and numerous pyroninophilic cells, possibly plasmablasts. (The last three sections were kindly sent to us by Dr. Mary Wilmers, Dr. S. A. Doxiadis and Dr. Grover Hutchins).

THYMIC NEOPLASIA—THYMOMAS

Thymomas are benign or locally invasive tumours which are of interest in being associated with certain extra-thymic diseases of probable auto-immune character: the predominant cell type tends to be correlated with the nature of the extra-thymic disease. The thymoma may be (a) *epithelial*, (b) *spindle-celled*, the cells forming compact masses or whorls resembling fibrous tissue, (c) *lymphocytic* or (d) *lympho-epithelial*, wherein epithelial cells and lymphocytes are more or less equally represented (Schmid *et al.*, 1965).

Myasthenia gravis is the disease most frequently associated with thymoma. Thus 15–30 per cent of all thymomas are complicated by myasthenia gravis and, taking all cases of myasthenia gravis, thymoma is present in 15–30 per cent (Castleman, 1955; Fisher, 1964). Thymomatous myasthenia gravis, in contrast with non-thymomatous myasthenia gravis, occurs in older subjects, is a more severe disease, is less responsive to treatment with prostigmine or by surgical thymectomy, is associated with a higher incidence of positive serological reactions to muscle, and the serological reactions more often persist after thymectomy. Moreover, in a small series, thymomatous myasthenia gravis was about eight times more often associated with myositis and myocarditis than was non-thymomatous myasthenia gravis (Genkins *et al.*, 1961).

Bone marrow aplasia with thymoma has been documented in at least 50 reported cases from various sources. There were 32 females with a mean age of 60 years and 18 males with a mean age of 58 years, the age range being 20–77 years. The tumour was

spindle-celled in 20 cases, a lympho-epithelioma in 12, lymphocytic in four and of undefined structure in the remaining 14. Myasthenia gravis coexisted in nine cases, hypogammaglobulinaemia in five and diabetes mellitus in four. Severe anaemia was present in all cases and, in addition, thrombocytopenia was present in 18 and leucopenia in 12. The essential lesion in the bone marrow was pure erythroid hypoplasia, but there was general aplasia, initially or eventually, in 12 of the cases. The L.E. cell test was positive in two cases and the Coombs' test was also positive in two cases. The precise relationship between the thymoma and the bone marrow disorder is uncertain since the tumour clearly preceded the anaemia in only 17 of the 50 reported cases, and thymectomy produced a significant remission in only four of the 29 patients in whom it was performed.

Adult acquired agammaglobulinaemia has been reported as coexisting with thymoma in 14 cases—ten females of mean age 51 years, and four males of mean age 62 years—and it has been stated that 5–10 per cent of cases of adult agammaglobulinaemia will be associated with a thymic tumour (Comings, 1965; Peterson, Cooper and Good, 1965). An associated bone marrow deficiency was present in five of the 14 cases. The tumour was spindle-celled in eight cases, epithelial in two, and lympho-epithelial in two. The essential cause of adult agammaglobulaemia is unknown, and no clear cause-and-effect relationship between thymoma and plasma-cell aplasia has been discovered. However it is of interest that relatives of cases of acquired agammaglobulinaemia have a significant incidence of rheumatoid arthritis and other diseases of auto-immune character, and auto-immune serological reactions (Fudenberg, German and Kunkel, 1962; Wolf, Gokcen and Good, 1963).

Myositis of one form or another has been reported in nine cases of thymoma, four of whom had an associated myocarditis; one had myocarditis only, together with a positive L.E. cell test, and one had dermatomyositis (Klein *et al.*, 1964). Systemic

lupus erythematosus has been reported in association with thymoma in three cases, and the L.E. cell test was positive in an additional three cases wherein the typical features of lupus erythematosus were not present. There are references to single cases of Sjögren's disease, and of rheumatoid arthritis, in associa-

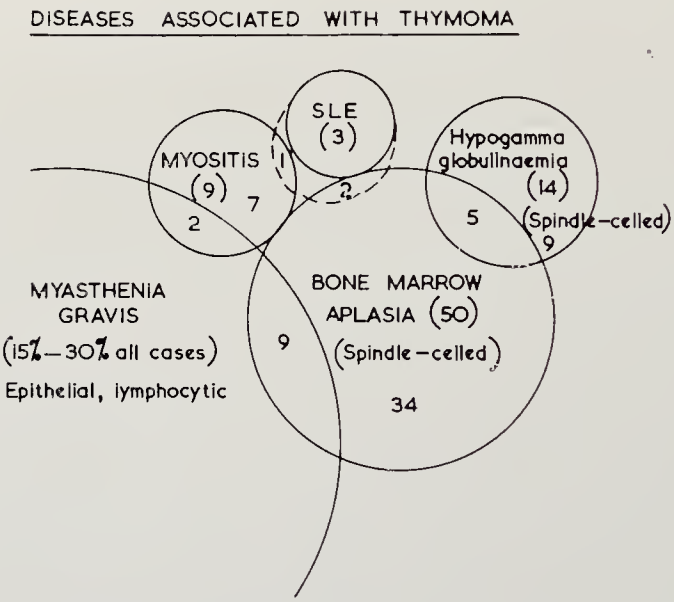

FIG. 7. Diagram showing interrelationship of diseases associated with thymoma. Numbers in brackets are total numbers of reported cases in that disease category. Numbers without brackets in the overlapping circles indicate cases where two conditions coexisted. Thymoma was associated with three cases of systemic lupus erythematosus (SLE), and the L.E. cell test was positive in three additional cases, as shown by the dotted circle. The predominant histological type of tumour is shown for the three major diseases.

tion with thymoma (Lattes, 1962), and there is a report of a thymoma associated with hyperglobulinaemic purpura wherein removal of the thymus effected improvement (Birch *et al.*, 1964).

The frequency with which thymoma is associated with extrathymic disease, either singly or in combination (Fig. 7), does not indicate coincidence or chance; thus we must accept that some

subtle interrelationship exists between thymoma and the various diseases associated with it. Myasthenia gravis, which is the main fellow-traveller with thymoma, can now be classified as an auto-immune disease, and thymoma has been associated with either frank lupus erythematosus, or a positive L.E. cell test, in six reported cases. Thus, an auto-immune process appears to be related in some way to this association between thymoma and extra-thymic disease.

INTERPRETATION OF THYMIC LESIONS AND AUTO-IMMUNE DISEASE

The nature of the association between thymic lesions, whether dysplasia or neoplasia, and auto-immune disease is uncertain, but three possibilities may be considered.

The *first possibility* is that lympho-epithelial proliferation in the thymus is the direct cause of the auto-immune process, either by the production of a humoral agent of auto-antibody nature, or by the seeding of self-reactive immunocytes to the periphery. However, it may be noted (a) that the epithelial type of tissue present in these thymic lesions is not that which is normally associated with antibody production, (b) that the onset of thymoma and the extra-thymic disease is often quite unrelated in time, with the extra-thymic disease even developing after excision of the thymus, and (c) that excision of the thymus often neither cures the associated disease nor influences the activity of the serological manifestations (Osserman and Weiner, 1965; Mackay and Smalley, 1966).

The *second possibility* is that abnormal cellular activity, for example thymoma, produces damage and/or functional abnormality in the remaining thymic tissue, and so interferes with the normal thymic function of preventing the emergence of self-reactive clones of lymphoid cells in the thymus. In support of this, it has been consistently observed that the "normal" thymic tissue adjacent to thymomas contains germinal centres (Lattes, 1962) (Fig. 8).

The *third possibility* is that an auto-immune process is the primary event, and germinal centres, epithelial hyperplasia and eventually tumour formation in the thymus are secondary reactions to auto-immune damage to the gland. The target auto-antigens would be skeletal and cardiac muscle in cases with myasthenia gravis and myositis, a bone-marrow stem cell in

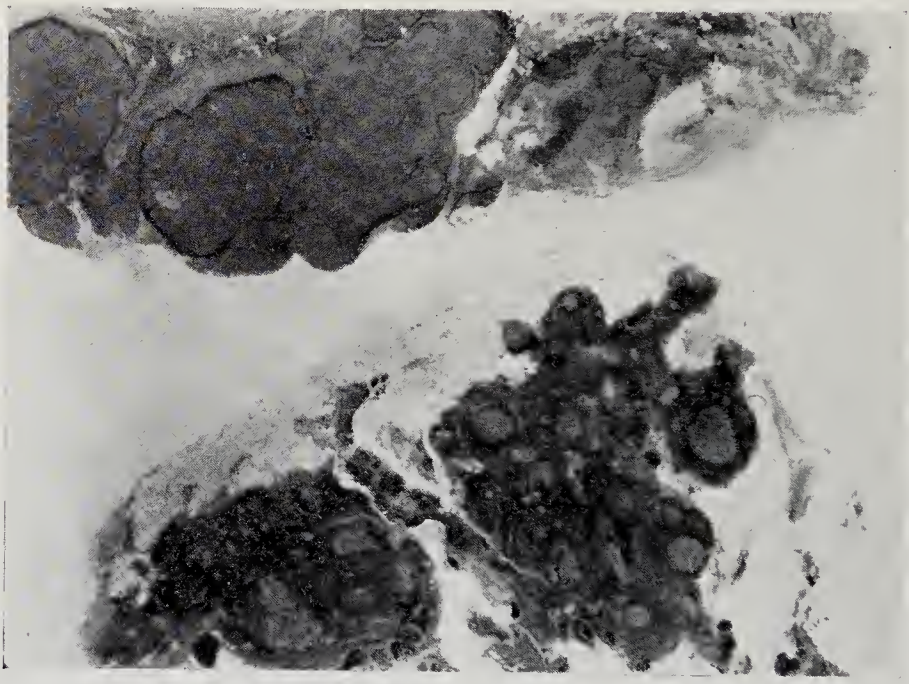

FIG. 8. Section of thymus from case of myasthenia gravis showing epithelial thymoma above and diffuse germinal centre development in adjacent thymus below. Papanicolaou. (× 10).

cases with marrow aplasia, and presumably some agent or cell concerned in plasma-cell development in agammaglobulinaemia. This concept implies that a specific relationship must exist between the target auto-antigen and thymic epithelium: a forbidden clone is formed capable of interacting with an antigen common to thymic epithelium on the one hand, and on the other to a muscle component and/or a bone-marrow stem cell

and/or a plasma-cell precursor. The forbidden clone, on lodging in the thymic medulla, is stimulated to proliferate, perhaps as a germinal centre. This proliferation could result in (a) the production of further immunocytes capable of reacting with thymic epithelium, and (b) continuing hyperplasia of thymic epithelial cells analogous to the proliferation of host cells in graft-versus-host reactions.

SUMMARY

Pathological changes in the human thymus comprise aplasia, hyperplasia, dysplasia and neoplasia. *Primary aplasia* determines the rare lymphopenic subtype of infantile agammaglobulinaemia. *Hyperplasia* is present in thyrotoxicosis: thyroxine may be a natural stimulus to thymic activity. *Dysplasia* includes (a) germinal centre formation in the medulla, as seen in myasthenia gravis, and (b) lymphoid depletion with spindle-epithelial cell aggregates and germinal centre formation in the medulla, as seen in lupus erythematosus. *Neoplasia* refers to benign thymomas, many of which are associated with extra-thymic diseases possibly auto-immune in nature.

There is a subtle interrelationship between thymic lesions and auto-immune disease: the thymic lesions may in some way be a cause of the associated disease, or the thymus may be a preferential target organ in certain auto-immune conditions.

ACKNOWLEDGEMENTS

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DISCUSSION

Burnet: Dr. Mackay has given us an extremely clear picture of the thymus and auto-immune diseases in man. Dr. Irvine, would you tell us something about the thymus and thyroid disease?

Irvine: My colleagues and I (Gunn, A., Michie, W., and Irvine, W. J. [1964]. *Lancet*, **2**, 776; Irvine, W. J., and Sumerling, M. D. [1965]. *Lancet*, **1**, 996) have studied the thymus in patients with thyroid disease in two ways. First, it is a simple matter to obtain a biopsy of a cornu

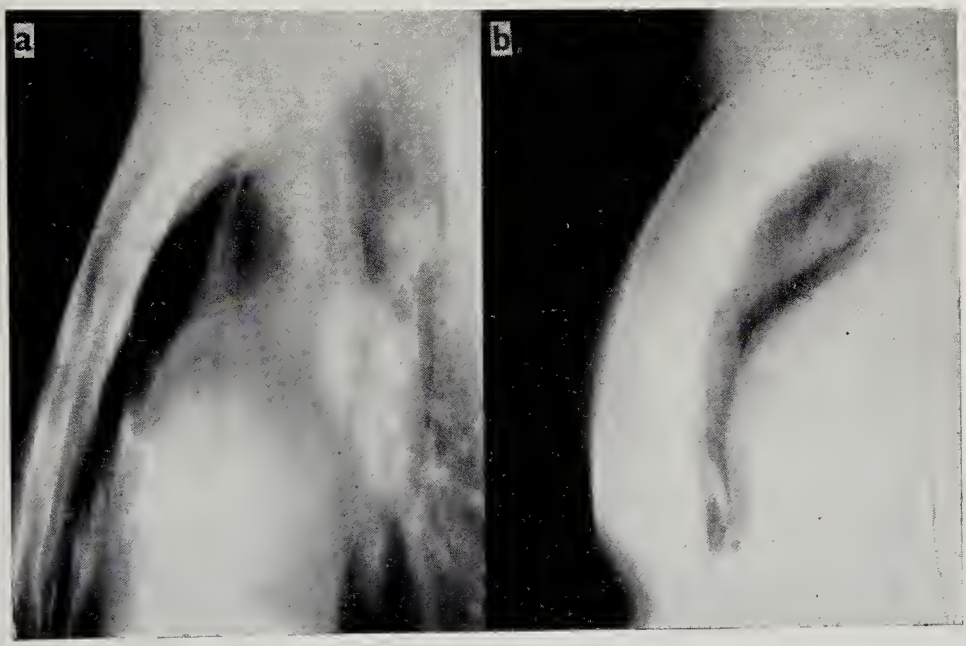

FIG. 1 (Irvine). Lateral radiographs (tomograms) of the chest using the technique of pneumomediastinography to demonstrate the thymus. The cross-sectional area of the thymus shadow in the central plane was estimated by planimetry. (a) Thyrotoxic female aged 36 years with negative serology. Thymus size, 2·6 cm.^{2} (normal). Note the two cornua of the thymus. (b) Thyrotoxic female aged 42 years with high titre of thyroid microsomal antibody (complement fixation titre = 256), moderate titre of gastric microsomal antibody (complement fixation titre = 32), low titre of antibody to thyroglobulin (tanned cell haemagglutination titre = 25) and negative test for antibody to intrinsic factor. The thymus shadow, 18 cm.^{2}, is considered to be enlarged. (From Irvine, W. J., Davies, S. H., and Sumerling, M. [1966]. *In* Proc. 5th Int. Thyroid Conf. Rome, May, 1965. Current Topics in Thyroid Research, p. 773. New York: Academic Press Inc.)

of thymus during partial thyroidectomy. We have found a correlation between the presence of germinal centres in the thymus and lymphoid follicles in the thyroid, but no correlation between the presence of thyroid antibodies in the serum and germinal centres in the thymus. Our second method of studying the thymus is by pneumomediastino-

graphy. Two examples of the radiological findings are shown in Fig. 1. Figure 2 summarizes the results of the thymus X-ray studies in patients with thyroid disease. The sera of the patients were examined for anti-

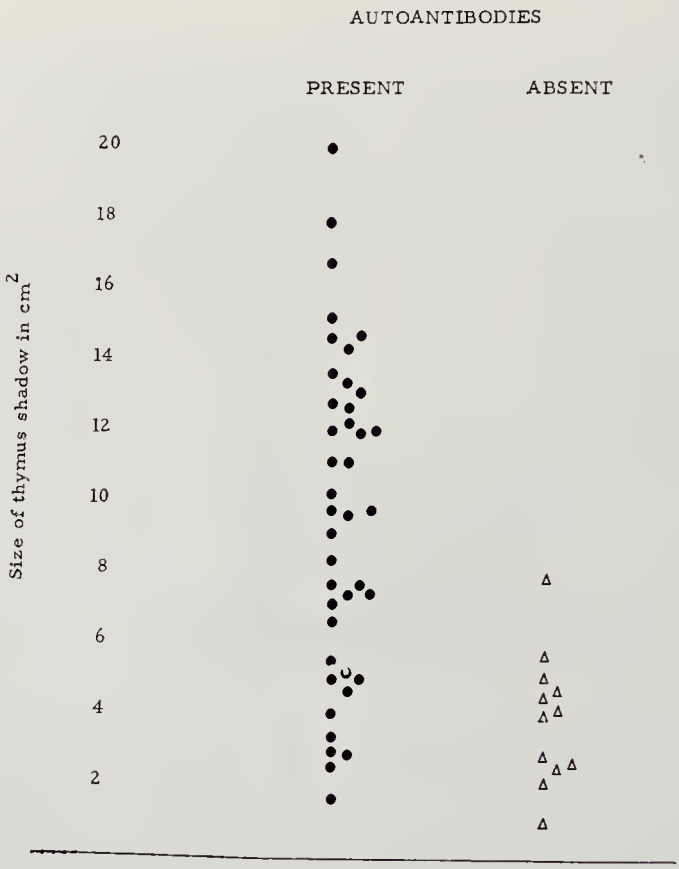

FIG. 2 (Irvine). The thymus in thyroid disease. The cross-sectional area of the thymus shadow in the central plane was determined by pneumomediastinography in 52 patients with thyroid disease. The size of the thymus is compared in patients with positive thyroid or gastric serology and in patients with no serological evidence of auto-immunity. (From Irvine, Davies and Sumerling, 1966, *loc. cit.*)

bodies to thyroid and to stomach, and for rheumatoid factor and antinuclear factors. The vertical axis represents the cross-sectional area of the thymus in the sagittal plane. There is a fair correlation between evidence of auto-immunity and thymic enlargement.

There are difficulties in studying the human thymus *in vivo*. There is the possibility of sampling error in the thymic biopsy, and the radiological technique, while delineating the thymus very clearly, provides no information about the thymic content of lymphoid tissue, connective tissue or fat. However, since we found no correlation between radiological evidence of thymic enlargement and generalized obesity, it seems reasonable to assume that an enlarged thymic shadow on X-ray may represent thymic hyperplasia, either in the past with subsequent replacement by fat, or in the present. It is encouraging, Dr. Mackay, that you found a relationship between the X-ray findings and thymus weight.

The level of circulating thyroxine may influence thymic size but I do not think this can be the sole explanation of our findings. All the patients were euthyroid at the time of study, some had a history of hyperthyroidism and others of hypothyroidism, and others with thyroiditis had remained euthyroid throughout. It seems that the thymus is involved, either directly or indirectly, in the phenomenon of autoimmunity in this group of adult patients.

Mackay: My evidence that hyperthyroidism causes thymic enlargement was derived from Hammar's data (Hammar, J. A. [1929]. *Z. mikrosk. -anat. Forsch.*, **16**, 733), and from Warner's experimental observation (Warner, N. L. [1964]. *Aust. J. exp. Biol. med. Sci.*, **42**, 401) that thyroxine increased the size of the cortex of the thymus in fowls. Thus I disagree with Dr. Irvine over the cause of thymic enlargement in thyroid disease.

I would like to underline what Dr. Irvine has said about the problems of using X-ray methods for diagnosing thymic enlargement in older people, because, with advancing years, this technique becomes less reliable as the thymus is progressively occupied by fat. We reserve our interpretations of this examination to younger patients.

Metcalf: An interesting thymic tumour is produced by certain sublines (SV10) of the polyoma virus. This is a lympho-epithelial, or an almost pure epithelial, tumour. Dr. Law (Law, L. W., *et al.* [1964]. *In* The Thymus: Wistar Institute Symposium Monograph No. 2, p. 105, ed. Defendi, V., and Metcalf, D. Philadelphia: Wistar Institute Press) has shown that transplants of this C3H tumour—a very slowly growing and, in fact, a regressing tumour—induce or restore immuno-

logical competence in neonatally thymectomized ($C_{57}BL \times C_3H)F_1$ recipients. This means that at least some of the tumour cells were functional and produced a factor or factors which restored competence. Some of the human cases with thymic tumours similar in appearance to the ones described by Law may also, therefore, be in a functional hyperthymic state. One wonders if in this state there might be not only excess reactivity of the normally competent cells, but also abnormal reactivity in some of the competent cells. In other words, is auto-immune reactivity sometimes produced in this way?

Burnet: Some of the thymuses we have examined, particularly in old hybrids of NZB and AKR strains of mice, show lympho-epithelial lesions looking like the lesions Law has described; they certainly contain large quantities of epithelium.

Strauss: Dr. Mackay, I am impressed with Dr. Goldstein's and your novel approach to the study of the histopathology of the thymus (Goldstein, G., and Mackay, I. R. [1965]. *Aust. J. exp. Biol. med. Sci.*, **43**, 381). This approach might help us in an attempt to study the life history of the lesions in the thymus in myasthenia gravis and lupus erythematosus. As far as I know this has not been done before, and in myasthenia at least there is sufficient histopathological material available for such a study. It might be possible to correlate thymic histology with the duration of the clinical disease in patients. Although we cannot study any one patient serially in any realistic way this approach might enable us to think about the changes in the thymus in terms of a continuum in relation to the clinical disease.

Burnet: Dr. Strauss' findings that there seem to be two groups of patients with thymomas add indirect confirmation to the idea that we can divide human auto-immune, or "possibly" auto-immune, diseases into two groups. In one we are primarily concerned with young women from adolescence to thirty years of age, in the other—the rheumatoid arthritis group—the disease becomes more common with advancing age. There may be a hint here that the thymus could be concerned in two directions: as a source of pathogenic cells in the young group and, through the disappearance of some control, with thymic atrophy in later life.

Good: We can now attempt to classify immunological deficiencies in man on a pathogenetic basis and show that many of these diseases

appear to be disorders of just one of the two forms of lymphoid tissue—the thymus and the bursa and their associated systems. Such a classification provides a new view of malignancies of the lymphoid system in man and shows that, as in the mouse and chicken, these diseases may involve primarily one only of the two lymphoid systems. Our classification, based on a study of over eighty of our own patients with various immunological deficiencies and on many cases from the literature, is shown in Table I.

If we look at the Bruton type of agammaglobulinaemia (Bruton, O. C. [1952]. *Pediatrics*, **9**, 722) in the perspective of the two-component system we see evidence for such an organization in man. These patients are similar to chickens with intact thymus and thymus-dependent peripheral lymphoid system but absent bursa and bursa-dependent lymphoid system. The clinical, immunological and histological features of this syndrome are tabulated in Table I.

The features of the Swiss type of agammaglobulinaemia, first described by E. Glanzmann and P. Riniker (1950. *Annls paediat.*, **175**, 1), are also shown in the table. In patients with this syndrome the maturation of the lymphoid system seems to be arrested at the stage of initial development of the epithelial thymus. Both the thymus-dependent and the bursa-dependent lymphoid systems have failed to develop, either because the first step—thymic development—is necessary for all subsequent stages of development of the lymphoid system or because a precursor cell essential for thymic development is also a precursor for bursal development.

Patients with the thymic alymphoplasia described by D. Gitlin and J. M. Craig (1963. *Pediatrics*, **32**, 517) and D. Gitlin, F. S. Rosen and C. A. Janeway (1963. *Pediatrics*, **32**, 517; 1964, *Pediatrics*, **33**, 711) are very similar to patients with the Swiss type of agammaglobulinaemia. The Gitlin and Craig alymphoplasics seem to have minimal development of the thymus and the thymic system of cells as well as failure of development of the immunoglobulin-producing plasma cells and germinal centres.

Patients with ataxia telangiectasia (Louis-Bar, M. [1941]. *Confinia Neurol.*, **4**, 32: Boder, E., and Sedgwick, R. P. [1958]. *Pediatrics*, **21**, 526) present still another anomaly: a considerable number of these patients, if they survive, develop either Hodgkin's disease, lympho-

TABLE I (Good) IMMUNOLOGICAL

Disease	*Inheritance*	*Number of Personal Cases*	*Immunological parameters, including immunoglobulins, antibody production, homograft, rejection, delayed allergy, graft-versus-host response*
Bruton type agammaglobulinaemia	Sex-linked recessive	30	Immunoglobulins, antibody production absent, cellular immunity normal
Swiss type agammaglobulinaemia	Simple autosomal recessive	5	All defective
Thymic alymphoplasia (Gitlin, Rosen, Janeway)	Sex-linked recessive	4	All defective
Thymic alymphocytosis (Nezelof, Allibone, Lee)	Probably simple autosomal recessive	0	Immunoglobulins normal or near-normal, homograft immunity, delayed allergy defective
Thymic alymphoplasia (DiGeorge)		0	Immunoglobulins normal, cellular immunity defective
Thymic alymphoplasia and alymphocyosist		0	All findings as in DiGeorge's syndrome, but parathyroid function normal
Ataxia telangiectasia (Louis-Bar, Boder, Sedgwick)		10	γA absent in 50–60%, γG low in 5%, feeble antibody response to weak antigens, normal antibody response to strong antigens
Dysgammaglobulinaemia (Rosen, Gitlin, Janeway)		2	γG absent, γM 3–4 times normal, total immunoglobulins normal, immunological parameters deficient
Aldrich's syndrome	Sex-linked, recessive	15	γG, γA normal, γM slow development, isohaemagglutinins absent, delayed allergy defective, antibody production normal except to allogenic and xenogeneic red blood cells
Syndrome of Heremans, Rockey, Hanson, Kunkel		1	γM, γG normal, γA absent, antibody production, etc., normal
Sporadic idiopathic hypogammaglobulinaemia (Good)	? simple autosomal recessive	10	γG low (100–300 mg. %), γM absent, antibody production defective, delayed allergy normal
Agammaglobulinaemia with benign thymoma		3	γM, γA absent, γG low (< 100 mg. %), antibody production defective, homograft rejection defective
Agammaglobulinaemia with follicular lymphoma		2	Immunoglobulins absent or low, delayed allergy normal
Acquired agammaglobulinaemia (Wollheim)	? simple autosomal recessive	14	Immunoglobulins absent or low

Table I continue

DEFICIENCY DISEASES IN MAN

Involvement or failure of development of central lymphoid tissue							
Thymic System			*Bursa-equivalent system*				
Thymus	*Small lymphocytes*		*Tonsils*	*Follicles tonsillar*	*Peyer's patches*	*Plasma cells, and germinal centres*	*Additional features*
	Blood	*Tissues*					
Normal	Normal	Normal	Lympho-epithelial development absent	Absent	Follicles absent	Absent	
Development deficient, no descensus or Hassall's corpuscles. Few mg. epithelial tissue, no cortex or medulla	Absent	Absent	Lympho-epithelial development absent		Follicles absent	Absent	
Development arrested, Hassall's corpuscles absent, weight < 2 g., no cortex or medulla	Low	Low		Absent	Follicles absent	Absent	
Poorly developed (as in Swiss type agammaglobulinaemia)	Absent	Absent	———	Not studied	———	Normal	
Absent	Absent	Absent	Normal	Normal	Normal	Normal	Failure of development of parathyroid glands with clinical hypoparathyroidism.
——— All findings as in DiGeorge's syndrome, but parathyroid function normal ———							
Development arrested, Hassall's corpuscles absent, no cortex or medulla	Low	Low	Under-developed	Normal	Not studied	Normal	Cerebellar ataxia, sinopulmonary infections. High incidence of lymphosarcoma.
Normal	Normal	Low	Normal	Normal	Normal	Normal (true Marschalko cells rare)	
Normal	Low	Low	Normal	Normal	Normal		Thrombocytopenia. Eczema. Poor resistance to infections
——— Not studied ———			———	Not studied	———		No clinical disease
Normal	Normal	Normal	———	Not studied	———	Low	
Benign spindle-cell thymoma	Normal	Low		Absent	Absent	Absent	Cases from literature: 18
Normal	Normal	Normal	Absent in one case	Hyper-plasia, pyrino-philic cells	Follicular hyper-plasia	Absent	Cases from literature: 12. ± Exudative enteropathy. ± Familial incidence of mesenchymal diseases.
Normal	Normal	Normal		Absent	Follicles absent	Absent	± Rheumatoid arthritis. No thymoma or follicular lymphoma

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TABLE I

Disease	*Inheritance*	*Number of Personal Cases*	*Immunological parameters, including immunoglobulins, antibody production, homograft, rejection, delayed allergy, graft-versus-host response*
Agammaglobulinaemia with myelomca		7	Normal immunoglobulins low, monoclonal γ-globulin excess antibody production defective, delayed allergy, homograft response normal
Agammaglobulinaemia with chronic lymphatic leukaemia			Immunoglobulins, antibody production, delayed allergy all defective
Hodgkin's disease		60	Immunoglobulins normal or high, antibody and homograft responses impaired, delayed allergy absent
Syndrome of Giedion, Scheidegger		2	γM, γA absent, γG low
Reticular-dysgenesis (de Vaal)		0	Not studied
Transient hypogammaglobulinaemia of infancy		7	Onset of synthesis of immunoglobulins delayed

sarcoma or lymphoreticular malignancy. This suggests that there may be a basic relationship between these malignancies and ataxia telangiectasia. The findings, as shown in the table, suggest a defect in immunological capacity particularly cellular immunity, a susceptibility to lymphoreticular malignancy and a frequent immunoglobulin defect involving primarily γA-immunoglobulin. We have examined the structure of the thymus at autopsy and biopsy in several of these patients. The gland is just like the thymus of an 18 or 19-day-old rabbit embryo, that is, it is primarily epithelial with only minimal evidence of lymphoid-cell development. It seems that these patients live their entire life with an embryonic thymus, leaving their peripheral lymphoid system underdeveloped and malfunctional and their central lymphoid tissue "bare". By contrast, most of these patients have normal plasma cells in their bone marrow and stimulated lymph nodes and spleen.

The immunological deficiency diseases of man first described by Nezelof and Allibone and their co-workers (Nezelof, C., *et al.* [1964]. *Archs fr. Pédiat.*, **21**, 897; Allibone, E. C., Goldie, W., and Marmion, B. P. [1964]. *Archs Dis. Childh.*, **29**, 26) are at present being studied in Sydney by Dr. Charles Lee (personal communication). I have seen a

continued

Involvement or failure of development of central lymphoid tissue							
Thymic System			*Bursa-equivalent system*				
Thymus	*Small lymphocytes*		*Tonsils*	*Follicles tonsillar*	*Peyer's patches*	*Plasma cells, and germinal centres*	*Additional features*
	Blood	*Tissues*					
Normal	Normal	Normal	———	Not studied	———	Excess, malignant	
May be involved by tumour	Excess, malignant	Excess, malignant					50% cases of chronic lymphatic leukaemia have agammaglobulinaemia or hypogammaglobulinaemia
May be involved by malignant process	May be low	Normal	Normal	Normal	Normal	Normal	
——— Not studied ———			——— Not studied ———			Few	
Vestigial or absent	Absent		——— Not studied ———				Granulocytes, platelets absent, red blood cells low.
——— Not studied ———			——— Not studied ———			Late development	

biopsy of lymphatic tissue from Dr. Lee's only patient and its appearance is entirely in keeping with the two-cell concept of lymphoid tissues. These patients have no lymphoid development of the thymus and no small lymphocytes in blood, lymph nodes, spleen or bowel. By contrast, the peripheral lymphatic tissue and the bone marrow contain abundant pyroninophilic large lymphocytes and plasma cells. As shown in the table, these patients usually have normal levels of γ-globulins. I consider that these patients have an underdeveloped thymus and thymus-dependent lymphoid-cell system with normal development and function of the immunoglobulin-producing system, the equivalent of the bursal system in the chicken.

There are now at least twenty different forms of immunological deficiency in man which have been recognized and partially defined. The most useful approach to understanding these defects is to postulate that in man as well as in the chicken there are at least two interacting but separate systems of lymphoid cells. A third interacting system may, perhaps, be the system of the macrophages.

I think that malignant disease of the lymphoid tissues as well as immunological deficiencies can be classified according to this new concept and I will now present my own speculations about this. The immuno-

logical deficiency of Hodgkin's disease, for example, suggests that this is a disease involving primarily the thymic system of cells. By contrast, myeloma is a disease in which the immunoglobulin-producing system is involved and the thymic system is left intact (see Table I). In twelve instances, follicular lymphomas have been associated with so-called acquired agammaglobulinaemia. Here, just as in chickens infected with RPL 12 virus, the immunoglobulin-producing follicular plasma-cell system is involved and not the thymic system. This abnormality might be viewed as an arrest of differentiation in the immunoglobulin-producing system at the blast-cell stage. From this perspective we may find new approaches to the analysis, treatment or even prevention of neoplasia of the lymphoid system in man.

We might also speculate that agammaglobulinaemic patients with benign thymomas may have secondary hyperplasia of their central lymphoid tissues to compensate for the deficiency in their peripheral lymphatic tissues.

We have studied two patients with acquired agammaglobulinaemia associated with follicular lymphoma who also had a remarkable family history of auto-immune disease and auto-immune serological reactions. Is it possible that the agammaglobulinaemia and failure of appropriate differentiation in the immunoglobulin-producing system is only one of several ways of expressing the faulty control residing in the immunoglobulin-producing system and that this is a genetically determined abnormality in these families?

O. M. DeVaal and V. Seynhaeve (1959. *Lancet*, **2**, 1123) have described a family containing infants with reticular dysgenesis. Such infants survive only a few days and are deficient in all the haematopoietic elements. Perhaps what fails to develop here is the multipotent lymphoid haemoblast (Jordan, H. E., and Speidel, C. C. [1930]. *Am. J. Anat.*, **46**, 335; Yoffey, J. M. [1964]. *Ann. N.Y. Acad. Sci.*, **113**, 867). This is the same cell that has been described by Dr. Ford and his colleagues (Ford, C. E., and Micklem, H. S. [1963]. *Lancet*, **1**, 359; Harris J. E. and Ford, C. E. [1964], *Nature, Lond.*, **201**, 884; Harris, J. E., *et al.* [1964]. *Nature, Lond.*, **201**, 886). If the lymphoid haemoblast fails to develop, neither thymic-dependent, immunologically competent cells nor any of the haematopoietic cells could be developed and survival would be impossible.

Study of these experiments of nature and their classification supports the concept that there are at least two separate central origins for peripheral lymphoid tissues, and that these must continue to differentiate peripherally. The ultimate functional integrity of these systems would be vulnerable to failure of central development, arrest of differentiation, or arrest of stimulus to proliferation either centrally or peripherally. Defects at any of these levels might be expected to give rise to disorders of immunological function.

THE SIGNIFICANCE OF AUTO-IMMUNE DISEASE†

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Auto-immunity has come a long way since it was considered the pipe-dream of certain wild-eyed visionaries (Gell and Coombs, 1963). The term *so-called* auto-immunity has now largely disappeared, particularly since Sir Macfarlane Burnet has contributed so extensively to the subject. Orthodox immunologists have now come into the field and see in auto-immunity a previously neglected area which may contain the answers to many significant questions. These include the role of immunocytes, imunological tolerance and its breakdown, the graft-versus-host reaction and the development of certain forms of leukaemia following an abnormal immunological reaction. In the course of this brief review of auto-immunity I will attempt to show that Burnet's concept of the forbidden clone has assumed far more than conceptual significance, and I will try to assess the significance of auto-immunity.

DEFINITION AND HISTORY

Auto-immunity may be defined as an abnormal immunological reaction against self-antigens, altered or unaltered. It represents an abnormal state in which the immune reaction, which is normally defensive and beneficial, has become harmful or

†Open lecture.

self-injurious. This abnormal reaction of self against self, which may be initiated by various techniques often becomes, eventually, a self-perpetuating abnormality in which there is a continuous reaction between self-immunocytes and self antigens, that is, *auto*-immunity.

This situation had seemed impossible because of interpretations given to Ehrlich's dictum—*horror autoxicus*—meaning that the body normally could not react against itself (Ehrlich and Morgenroth, 1899). Ehrlich did not make it clear if this might occur under exceptional circumstances. Shortly after this Donath and Landsteiner (1904) described what we might now call an experiment of nature—paroxysmal cold haemoglobinuria—which should have demolished Ehrlich's concept. In this syndrome autohaemolysis was demonstrated which could only be produced by mechanisms within the patient. The haemolysin reacted with the patient's own red cells in the cold and haemolysed them; that is, it was an *auto*haemolysin. Chauffard and Troisier (1908) and Widal, Abrami and Brulé (1908) extended these observations to describe haemolysinic anaemias and to show that haemolysins and auto-agglutinins were present in some cases of acquired haemolytic anemia. More than twenty-five years later, we rediscovered autohaemolysins and auto-agglutinins in acute haemolytic anaemia (Dameshek and Schwartz, 1938*a*, *b*) and began to use the term auto-immune haemolytic anaemia (Dameshek and Komninos, 1956). The concept spread to other haematological disturbances such as idiopathic thrombocytopenic purpura (Harrington *et al.*, 1951), combinations of this with auto-immune haemolytic anaemia (Evans *et al.*, 1951), and finally to systemic lupus erythematosus in which many abnormal immunological mechanisms were found (Dameshek, 1958). Auto-immune disorders were usually of obscure aetiology, usually showed a marked female-male preponderance, a rather high familial incidence and were self-perpetuating. Systemic symptoms including fever, weight loss, night sweats and a high erythrocyte

sedimentation rate were usually present, together with hypergammaglobulinaemia, and often two or more immunological abnormalities.

Between 1940 and 1955 these disorders were described largely by clinicians and haematologists. Recently, the area of auto-immunity has been strengthened and perhaps even revolutionized through the active investigations of such immunologists as Witebsky (1965), Williams and Kunkel (1965) and Burnet (1959*a*).

THE DEVELOPMENT OF AUTO-IMMUNITY

Immunity was first thought to be a quiet, purely defensive mechanism. That an immunological reaction could be symptomatic and harmful was shown in the anaphylactic state and in those reactions which von Pirquet (1906) called "allergy". In auto-immunity the immune reaction may be occult, with only an abnormal protein or antibody in the patient's serum to indicate that an abnormality is present, or there may be indications of disease; this led Coombs and Gell (1963), in line with Pirquet's concepts, to advocate the term auto-allergy rather than auto-immunity.

Whatever one calls this condition, there can be no doubt (a) that immunological reactions against self-components can occur and (b) that these may be harmful and lead either to such specific abnormalities as auto-immune haemolytic anaemia, idiopathic thrombocytopenic purpura, thyroiditis, or to disorders with several manifestations such as systemic lupus.

During 1940 to 1955, the sceptics asked: "Since you call these abnormal proteins in the blood auto-antibodies, where are the antigens?" In the last decade, these questions have been at least partially answered. It has become reasonably clear that auto-immunity may develop either because antigens have been sequestered in inaccessible areas or have become altered, or

because unaltered self-antigens have encountered a defective immunocyte system. Two broad possibilities for the development of auto-immunity exist: (a) an antigen-oriented cause and (b) an aberrant recognition system by abnormal immunocytes.

ANTIGEN-ORIENTATED HYPOTHESES

It is reasonably clear that there exist in the body certain unaltered, sequestered or inaccessible antigens such as those in the lens of the eye and in spermatozoa, thyroglobulin, and perhaps certain elements of the central nervous system. These may never reach the circulation and thus never come in contact with the adult immunocyte system. It is assumed that during foetal life these antigens were either not present, not fully developed or even then inaccessible so that the usual mechanism for developing tolerance to them was not achieved. In adult life a leakage of such antigens is always possible, through infection, trauma, surgery and so on. Under these circumstances antigenic material may reach the system of immunocytes with potentially disastrous results, for they may not be recognized as self and may induce the development of new antibody by self-immunocytes. This mechanism has been experimentally reproduced for the lens (Halbert, Manski and Ehrlich, 1965), thyroglobulin (Rose *et al.*, 1965), spermatozoa (Feltkamp *et al.*, 1965) and elements of the central nervous system (Waksman, 1965) with the development in the experimental animal of ophthalmitis, aspermatogenesis, thyroiditis and allergic encephalomyelitis respectively. These antigens are organ specific: to the system of immunocytes the reaction is against what is fundamentally a new antigenic constituent.

The reactions against altered self-antigenic constituents are less well-defined. Most attempts to produce auto-immunity against such constituents have failed, although with Freund's adjuvant and with hetero-antigens, auto-antibodies and cross-reacting

antibodies are obtained (Rose, Kite and Doebbler, 1962). In certain cases of acute myeloproliferative disorders, for example the Di Guglielmo syndrome in which abnormal or neoplastic nucleated red cells are present, auto-antibodies are sometimes discovered (Finkel *et al.*, 1966). Certain antigens—of the red cells in this syndrome—could be radically altered in the neoplastic process and thus induce the development of a self-perpetuating auto-immune reaction. The development of blood group changes in some cases of leukaemia would be in line with this (Renton *et al.*, 1962).

IMMUNOCYTE-ORIENTED HYPOTHESES

Burnet (1959*b*) developed the concept of the forbidden clone. This is a group of immunocompetent cells derived from the asexual replication of a single cell which, in some way, perhaps by mutation, has emerged and is capable of reacting immunologically with self-antigens. This may be considered as the reverse of the normal immune reaction. Instead of normal immunocytes disposing of foreign antigens, abnormal immunocytes react against self-antigens with the development of abnormal antibodies.

The immunocytes are a highly specialized complex of cells comprising (a) phagocytic macrophages, (b) lymphocytes which, in response to antigen, produce (c) haemocytoblasts which leads to the development of (d) further (committed) lymphocytes and plasmacytes (Dameshek, 1963). The immunological reaction may be considered as a proliferative response in which the entire immunocyte complex is involved: the macrophages phagocytose foreign material which contains antigenic substances and digest it (Roberts and Haurowitz, 1962), this antigenic material or the information derived from it is picked up by small lymphocytes which gather about the macrophages (Sharp and Burwell, 1960), and these lymphocytes, in response to antigen, dedifferentiate into

primitive cells (immunoblasts) (Tanaka *et al.*, 1963) which possess large numbers of ribosomes. Proliferation then ensues. It is not known if immunoblasts lead to the formation of both plasmacytes and lymphocytes, but there can be no doubt that the plasmacytes produce γG-immunoglobulin (Schoenberg, Rupp and Moore, 1964). On the other hand, the site of γM-immunoglobulin production (this is the first immunoglobulin produced in the immune reaction) is not clearly defined; the large ribosome-laden immunoblasts and the early lymphocytes may provide the answer. Following the immune reaction, memory cells—almost surely the ubiquitous lymphocytes—remain; these have the capacity to react quickly to the same antigen if it returns (Gowans and McGregor, 1965). The small, apparently mature, lymphocyte has thus achieved a central importance in the immune reaction.

Our interest in the possibility that an abnormal immunocyte complex—the forbidden clone—could be responsible for auto-immune reactions arose from our observation of the frequent coexistence of chronic lymphocytic leukaemia and the various auto-immune disorders, particularly haemolytic anaemia (Rosenthal *et al.*, 1955). The lymphoproliferative disorders were frequently associated with auto-immunity, whereas such an association with the myeloproliferative disorders was uncommon. We have concluded that the presumably abnormal lymphoid system of chronic lymphatic leukaemia could function immunologically even though it was abnormal, and could thus react with normal self-antigens leading to auto-immunity (Dameshek and Schwartz, 1959).

How do abnormal clones develop? Burnet has emphasized somatic mutation, particularly in such actively proliferating tissues as the lymphoid system. Other possibilities include the inheritance of abnormal clones and their emergence as functional units, perhaps in middle age, or the transplacental transfer of abnormal lymphocytes from mother to foetus (Desai and

Cregar, 1963). The lack of certain controlling mechanisms for such cells as the lymphocytes may be important, especially in hypogammaglobulinaemia in which auto-immune disturbances are common. The development of forbidden clones in the thymus may also be important (Burnet, 1962).

Auto-immunity might be produced in various ways: as the result of sequestered antigens becoming accessible to the host's immunocyte system; through the continued production of altered antigens which are no longer recognized as self-constituents; or through the presence or development of abnormal clones of immunocytes. The latter possibility seems to fit most of our clinical cases of auto-immunity, whether these are idiopathic (so-called) or occur in the course of chronic lymphocytic leukaemia or lymphosarcomatosis.

These inferences have been given experimental justification in the graft-versus-host reaction described by Oliner, Schwartz and Dameshek (1961) in runt disease. Parental spleen cells A, when injected into F_1 hybrids containing A and B cells, were tolerated by the host's immunocytes but the A immunocytes of the graft reacted with the B antigens and induced an immunological response. As a result, the graft-versus-host reaction developed, comprising auto-immune haemolytic anaemia, thrombocytopenia and lesions suggestive of lupus erythematosus and rheumatoid arthritis. It was evident that half of the host antigens were foreign to the graft's immunocytes. This reaction was in many ways similar to auto-immune disease in lymphocytic leukaemia, and possibly to many of the idiopathic auto-immune disturbances such as systemic lupus erythematosus.

Much of the mystique of the forbidden clone depends upon its tolerance, for variable periods, by the host. The host tolerates this foreign graft (of immunocytes) and the graft tolerates the host but this tolerant situation may be abrogated at any time. For example, in both clinical and experimental circumstances ionizing radiation can result in severe auto-immune disorders.

We have shown that auto-immune reactions may develop in some patients with chronic lymphocytic leukaemia treated by total-body irradiation or large doses of alkylating agents (Dameshek and Gunz, 1964). In recovered cases of the graft-versus-host reaction, as in the experimental mouse, Schwartz and Beldotti (1963) have shown that a small dose of total-body irradiation can trigger off another fatal attack of runt disease. In both the clinical and the experimental situations one may speculate that the patient or mouse, surrounded by and tolerant of forbidden clones, loses this tolerance and develops manifestations of auto-immune disease.

The exact mechanism by which this lack of tolerance develops is not known, but one infers that there is a loss of the recognition mechanism of the clonal immunocytes with the development of a violent immunological reaction, harmful to the host.

IMMUNOPROLIFERATIVE DISORDERS

Table I shows the immunological abnormalities, including auto-immune disease, which occur so often in the lymphoproliferative disorders.

Auto-immune disorders may occur at any stage of the chronic lymphoproliferative diseases and may even be seen before there are any indications of leukaemia or lymphosarcomatosis. The latter two diseases are of particular interest because they suggest the theoretical possibility that auto-immune disease—a presumed clonal disturbance—may lead to leukaemia.

The many relationships of auto-immune disease to certain forms of leukaemia and the often difficult task of differentiating an auto-immune reaction from a leukaemic one have been a recurrent theme in our thinking. These relationships are to be found in chronic lymphocytic leukaemia and lymphosarcomatosis, in the interesting relationships between cold haemagglutinin disease, macroglobulinaemia. and chronic lymphocytic leukaemia and in

infectious mononucleosis, which may be considered as a self-limited type of lymphocytic leukaemia. Of great interest are the cases of auto-immune disease, apparently idiopathic, terminating in leukaemia or lymphosarcoma. The apparently idiopathic auto-immune disorder, cold haemaglutinin disease, may end up as the leukaemic disturbance macroglobulinaemia (Ritzmann and Levin, 1962). Sjögren's syndrome, probably a complex auto-immune disease, may also terminate as leukaemia or lymphosarcoma (Talal and Bunim, 1963).

TABLE I

A CLASSIFICATION OF THE LYMPHOPROLIFERATIVE DISORDERS

	Disease	*Auto-immune manifestations*
	Infectious mononucleosis (self-limited)	Heterophile agglutination reaction Cold haemagglutinin cryoglobulins Auto-immune haemolytic anaemia Idiopathic thrombocytopenic purpura
Self-perpetuating	Chronic lymphocytic leukaemia	Auto-immune haemolytic anaemia Rheumatoid arthritis Idiopathic thrombocytopenic purpura
	Lymphosarcomatosis	Vascular purpura Thyroiditis Systemic lupus erythematosus and others
	Macroglobulinaemia	Auto-immune cold haemagglutinin disease
	Heavy chain disease	Auto-immune haemolytic anaemia

Patients with abnormal immunological reactions in the serum may eventually develop an immunoproliferative disorder. For example, a patient with a persistent spike of γG-immunoglobulin without plasmacytosis may eventually develop multiple myeloma (Osserman, 1959). Conversely, an individual with congenital or acquired hypogammaglobulinaemia may develop either auto-immune disease or a lymphoproliferative disorder. An individual with anergy could, perhaps, develop Hodgkin's disease, since anergy is found in some early cases of this disorder.

The neoplastic disorders involving the immunocyte complex (see Table I) are characterized by (a) a proliferation of one of the members of the immunocyte complex and (b) the presence of one or several aberrations of the immune reaction. The latter include various abnormalities ranging from hypo- to hypergammaglobulinaemia, from normo- to macroglobulinaemia, and from hetero- to auto-antibodies. In contrast, patients with multiple myeloma in whom there is a generalized proliferation of plasmacytes, show a sharp spike of either γG- or γA-immunoglobulin but almost never a reaction to a specific antigen or any features of auto-immunity. In the reticuloses—Hodgkin's disease and sarcoidosis—lymphopenia and a reduction in delayed hypersensitivity (Aisenberg, 1962)—even anergy—are commonly found and auto-immune reactions are uncommon. In the presumed functional abnormalities of immunoglobulin synthesis such as hypogammaglobulinaemia, the plasmacytosis is absent, but auto-immune disorders are common and there is a high incidence of leukaemia and lymphosarcoma (Good, 1959).

The differences between an auto-immune reaction and a leukaemic one may not be very great. If we assume that the auto-immune reaction is frequently based on the activities of an abnormal but tolerated clone of immunocytes, then it is possible that this clone may be continuously expanded by steady proliferation in response to continuous stimulation by normal antigens. If there is a block in plasmacytic proliferation, as may be presumed in congenital hypogammaglobulinaemia, lymphoid proliferation may become dominant and continue to expand. The leukaemic proliferation—at least of immunocytes—may be just an expanded although abnormal immunological proliferation.

This speculation has recently been fortified by animal observations and investigations as follows: Porter, Dixon and Larsen (1965) —Aleutian mink disease; Schwartz and Beldotti (1965)—graft-versus-host reaction; and Mellors (1966)—NZB mice with auto-immune haemolytic anaemia (see also this volume, pp. 410–412).

Experimentally, as well as on clinical grounds, there are indications that auto-immunity and immunocyte proliferation are closely related and may be simply different expressions of the same fundamental growth abnormality; leukaemia may be just an expanded form of abnormal immunological proliferation. The term leukaemia indicates a *mass* of proliferating and presumably neoplastic leucocytes that can be visualized because of its large and generalized bulk. A smaller mass of cells may only be visible microscopically but may be just as abnormal; that is, the cells in these two groups of disorders may be qualitatively the same although they are quantitatively different.

THE PLACE OF THE THYMUS

The thymus, neglected for two centuries, has come into prominence again in the past five years. Hewson (1777) showed that the thymus was concerned largely with maintaining "the cells with a central core" (lymphocytes) in the thoracic duct and the lymphoid organs. The gland, Hewson inferred, did this largely by what is now called its peripheralizing function. His experiments, and those of modern investigators, showed that lymphocytes from the thymus found their way into lymphatic channels and thence into the thoracic duct, lymph nodes and spleen. A circulation of lymphocytes between the thymus, thoracic duct, lymph nodes and spleen has been visualized and recently a hormonal effect of the thymus has been demonstrated (Miller, Osoba and Dukor, 1965).

Removal of the thymus is followed by lymphocytopenia and a reduction of immunological function (Miller, 1964). Because of its apparently primordial relationship to the lymphoid system and the lymphocytes, the thymus has become known as the chief organ of immunity. Without it in early life, there is little or no lymphocyte, lymph node or splenic development; without it, animals respond poorly to antigenic materials and may be considered to be immunological cripples.

The thymus, in contrast with other lymphoid organs, normally has no germinal centres, but in some auto-immune disorders, such as the haemolytic anaemia of the NZB mice (Bielschowsky, Helyer and Howie, 1959) and myasthenia gravis (Castleman and Norris, 1949), germinal follicles are present. This abnormality led Sir Macfarlane Burnet to speculate that the abnormal germinal follicles of the thymus might either be the active forbidden clones of auto-immunity or might represent the first step in the development of such clones. Unfortunately there is no direct evidence for this yet.

Good and co-workers (1965) have suggested that there are two centres directing immunity. These are the thymus, and the bursa of Fabricius in the chicken or an analogue of this lymphoid mass, possibly the appendix or the tonsil, in mammals. These workers have further suggested that the collar of lymphocytes around the germinal follicles is controlled by the thymus and the germinal follicle itself by the bursa or its equivalent and that the lymphocyte collar is responsible for cellular immunity, the germinal follicle for humoral antibody immunity. Good and his colleagues suggest that auto-immune and neoplastic lymphocytic disorders are mediated by overactivity of one or other of the systems of cells in the thymus (lymphoid collar or germinal centres) and thus controlled either by the thymus, or the bursa or its analogue. Further evidence for these attractive hypotheses is needed.

The thymus and myasthenia gravis are closely related. The evidence for this is the presence of thymic germinal follicles in most cases of myasthenia (Castleman and Norris, 1949), the presence of thymoma in many cases and antibody against thymic epithelial cells and muscle cells in some (Van der Geld *et al.*, 1963). The thymus is also clearly related to some cases of pure red cell anaemia in which a thymoma is often present; recent observations indicate that an auto-immune mechanism against erythropoietin exists in these cases (Roland, 1964). The relationship of the thymus to other auto-immune disturbances is not yet clear.

IMMUNOSUPPRESSIVE AGENTS AND AUTO-IMMUNITY

Because of the many similarities between auto-immune disorders and chronic leukaemia (self-perpetuating nature, continuous cellular proliferation, response to corticosteroids) the use of antileukaemic agents in supression of the immune process was considered. Schwartz, Stack and Dameshek (1958) demonstrated that suppression of the humoral antibody response could be achieved with 6-mercaptopurine. Schwartz and Dameshek (1959) showed that specific immunological tolerance could be induced by giving antigen simultaneously for a few days with the chemical. Other manifestations of the immune response, for example delayed hypersensitivity and the transplantation rejection phenomenon, could be modified considerably by antileukaemic agents. In some auto-immune disorders, for example haemolytic anaemia, 6-mercaptopurine was highly effective in aborting an attack (Schwartz and Dameshek, 1962), in others, such as systemic lupus erythematosus, the effects were inconstant, but at times—especially when large dosage was given—striking remissions could be established. This was most impressive in cases in which corticosteroids or other treatment had been ineffective or actually harmful. Other antimetabolites such as methotrexate, given intravenously, and chlorambucil were also found to be useful in some cases. These immunosuppressive drugs are simultaneously myelotoxic so that their clinical use is still largely experimental and must be carefully controlled. In some situations, such as transplantation, they have assumed definite importance.

THE SIGNIFICANCE OF AUTO-IMMUNITY

The most important aspect of auto-immunity is that it exists! It is now apparent that the body may have, or may develop, mechanisms whereby it can turn against itself either for good or for bad. The *abnormal* situation of auto-immune disturbance

reveals how important is the *normal* situation of tolerance of the body for its own antigens, through its stems of immunocytes. There may be advantages in a system which can recognize abnormal self-components and destroy them before they become neoplastic and of ecological importance. On the other hand, since the system of immunocytes "knows how" to do only one thing, which is to capture and eventually destroy what it considers foreign or not-self, it may react against previously inaccessible but *normal* tissues and thus do harm. Or it may react against altered antigens—constantly produced—and evoke a harmful reaction, as, perhaps, in rheumatoid arthritis. Even when immunocytes become neoplastic they may retain immunocompetence. In chronic lymphocytic leukaemia, for instance, the immunocytes react against the host-patient, because this is what they know how to do and because, presumably, they do not recognize normal self-antigens, and auto-immune disease develops. This most frequently takes the form of haemolytic anaemia, probably because the ubiquitous erythrocytes must come into contact with immunocytes more than do any other cells or tissues.

Auto-immune disease was first described in non-leukaemic disorders and designated idiopathic. Why it developed was mysterious but the concept of the forbidden clone has illuminated many of the problems. Burnet (1959*b*) speculated that clones of immunologically competent cells might emerge, presumably by mutation or possibly by depression, and react with normal antigens. Because such immunocytes regarded the self-antigens as foreign they could react against them immunologically. This concept was advanced at first without any experimental evidence. Bielschowsky, Helyer and Howie's discovery of auto-immune haemolytic anaemia as a genetic disturbance in a strain of NZB mice, and the demonstration of germinal follicles in the thymuses of these mice in Burnet's laboratory first suggested the possibility that the forbidden clones came from the thymus. It was later shown that the disorder could be passed from mouse to mouse

by injecting forbidden clones—spleen cells—into the animals (Holmes, Darrie and Burnet, 1961).

In our laboratory the graft-versus-host reaction has been used as the chief experimental model for demonstrating that genetically different, immunologically competent cells (from a single clone) can induce a reasonable facsimile of auto-immune disease (Oliner, Schwartz and Dameshek, 1961). These studies were confirmed in rats by Stastny, Steinbridge and Ziff (1963). The importance of the concept of the forbidden clone was not fully appreciated until very recently, when both NZB mice (Mellors, 1966) and the graft-versus-host reaction (Schwartz and Beldotti, 1965) revealed that (a) a genetically determined, presumed clone of auto-immune haemolytic-anaemia-producing cells eventually resulted in a generalized leukaemic disease and (b) an experimentally injected clone of spleen cells (immunocytes) induced, first, an auto-immune-like disorder and, eventually, leukaemic disease. These results can also be equated with the development of multiple myeloma lesions after diffuse hyperglobulinaemia in Aleutian mink disease (*vide supra*); in this disease, a forbidden clone of plasmacytes develops into generalized, leukaemic multiple myeloma.

These experimental observations can, finally, be equated with the experiments of nature seen in clinical practice, that is, with the development of leukaemic or lymphomatous disease *following* initial auto-immune disorders. Thus arose the concept that immunoproliferative disorders are neoplastic proliferations of immunocytes arising from abnormal groups of immunocytes, that is from forbidden clones. The concept of the forbidden clone has thus assumed increasing importance: a small clone may result in an abnormal auto-antibody; a larger, greatly expanded one, in leukaemia. The highly theoretical concept that some forms of leukaemia and related diseases are immunologically mediated requires further work, but is already valuable. It may lead to the idea that auto-immunity should be suppressed as

totally as possible. This is the rationale behind our use of the immunosuppressive drugs for these conditions. Although this work is still in its experimental stages some remarkable, and apparently completely suppressive, results have been achieved.

Overpreoccupation with the concept of the forbidden clone may have led to our minimizing the importance of the sequestered or inaccessible antigen (as in thyroiditis, sympathetic ophthalmia and aspermatogenesis) and the possibility of permanent alteration of antigen in some diseases. In the myeloproliferative disorders auto-immune reactions are common and it is possible that antigens, notably of red cells, may be altered in these diseases and evoke auto-immune reactions (Finkel *et al.*, 1966).

Finally, the large and burgeoning number of auto-immune disorders suggests to me that more are to come. Any tissue in the body or antigenic determinant may be the site of an auto-immune immunological disturbance. It is not, therefore, surprising that in recent years pernicious anaemia (Doniach, Roitt and Taylor, 1963), myasthenia gravis (Simpson, 1960) and even pure red cell anaemia (Roland, 1964) have been cited as auto-immune disorders.

The field of auto-immune disease needs further exploration but it needs, even more, careful pruning and nurture of what we already know. Watching the development of this field for more than a quarter of a century has been a thrilling experience for me; the developments in the next twenty-five years may be even more exciting.

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GROUP DISCUSSION

Šterzl: It is usual at scientific conferences for more questions to be asked than answered. I will therefore attempt to summarize the problems which have not been solved or discussed and on which experimental work in the future should be focused.

I would like first to discuss some aspects of the embryogenesis and physiology of the thymus. The significance of the two existing types of lymphocytes—thymic lymphocytes (thymocytes) of epithelial origin and peripheral lymphocytes of mesenchymal origin—is still unexplained. We do now have some data on the different properties of these two types of cell but their origins are still not fully settled. For example, in the studies demonstrating the epithelial origin of thymocytes, contamination of the epithelium with mesenchymal cells might have occurred (Clark, S. L. [1964]. *In* The Thymus in Immunobiology, p. 112, ed. Good, R. A., and Gabrielsen, A. E. New York: Hoeber). On the other hand, it has been observed that lymphocytes appear in the peripheral lymphatic tissue only after seeding-out of cells from the central lymphatic tissue, that is, of thymocytes (Auerbach, R. [1963]. *Natn. Cancer Inst. Monogr.*, **11**, 23). Further experimental embryological work as well as studies on thymic communications may help to solve this problem.

A series of studies on the migration of thymic cells has been done mostly under artificial conditions, for example, by the subcutaneous transfer of thymocytes (Harris, J. E., and Ford, C. E. [1964]. *Nature, Lond.*, **201**, 884). The migration of thymocytes from the thymus into the blood has been suggested (Fichtelius, K. E. [1960]. *Ciba Fdn Symp. Haemopoiesis: Cell Production and its Regulation*, p. 204. London: Churchill; Nossal, G. J. V., and Gorrie, J. [1964]. *In* The Thymus in Immunobiology, p. 288,

loc. cit.) but not yet proved. This may be partly due to our scanty knowledge of the lymphatic drainage of the thymus, although it is probable that the thymocytes reach the bloodstream through the lymphatic vessels and the thoracic duct. The inflow of cells to the thymus seems to be more definite. It has been established that macromolecular substances (antigens) can pass through the blood-thymus barrier (Sainte-Marie, G. [1963]. *J. Immun.*, **91,** 840). Plasmacytes demonstrated in the thymus are also thought to be cells that immigrate there from the periphery (Sainte-Marie, G. [1965]. *J. Immun.*, **94,** 172) and not cells of thymic origin as suggested by A. H. E. Marshall and R. G. White (1961. *Br. J. exp. Path.*, **42,** 379).

Another interesting study would be an investigation of the significance of thoracic localization of the thymus in mammals compared with animals at a lower phylogenetic level, for in these "lower" animals thymus analogues are connected almost exclusively with the gastrointestinal tract.

The main function of the thymus is thought to be the production of a humoral factor which influences proliferation of lymphatic tissues. New experimental models for quantitative estimations of this effect of the thymus are badly needed. Models which have already been studied, as well as thymectomy and the injection of thymic cells or extracts, include tissue culture in double diffusion chambers and the influence on intensity of effect of incorporating nucleic acid precursors into lymphatic cells. Although the results from these studies are not always fully significant, data on the stimulating effect of thymic products on lymphopoiesis are accumulating.

The thymus may have a positive effect on the proliferation of lymphoid tissue, but it may simultaneously have a negative effect on the differentiation of lymphocytes. Holub cultivated spleen tissue with thymocytes in double diffusion chambers (Holub, M. [1966]. *Folia microbiol., Praha*, in press) and found an enhanced proliferation of the lymphocytes but a decreased capability of these

cells to differentiate into antibody-forming cells. The specificity of the postulated thymic lymphopoietic factor must be considered. A lymphopoiesis-stimulating effect has been observed with extracts from mouse lymphoblasts (Duplan, J. F., Forschi, G. V., and Manson, L. A. [1962]. *Proc. Soc. exp. Biol. Med.*, **110**, 426) but, in contrast to this, a factor from peripheral mesenchymal tissue, the bone marrow, affecting thymic regeneration has also been described (Weymouth, P. P., *et al.* [1955]. *J. natn. Cancer Inst.*, **15**, 981).

We have discussed whether a hormone or a factor resembling an embryonic inductor is secreted by the thymus. I would like to suggest yet another possibility: when tissue is proliferating by mitosis it has definite needs for structural components out of which it can synthesize new nucleic acids, and lack of these components may decrease mitotic activity especially in growing organisms. Injections of thymidine (Grenlich, R. C., Cameron, I. L., and Thrasher, J. D. [1961]. *Proc. natn. Acad. Sci. U.S.A.*, **47**, 743) or other nucleotides can enhance mitotic activity. The significance of the thymus, which contains a large amount of nuclear material, may therefore be that it supplies trophic substances to the lymphoid tissues and that these substances are decisive during the period of maximal growth. The hypothesis that thymocytes function as donors of nucleic acids is not new and has been reviewed in detail (Kelsall, M. A., and Crabb, E. D. [1958]. *Ann. N.Y. Acad. Sci.*, **72**, 293).

The thymus cannot be considered without discussing its interaction with other important factors of the organism, especially the products of the endocrine glands. Professor Furth has described the action of testosterone on the thymus, but we should also investigate the relationship of the thymus to other endocrine products especially those that influence cell proliferation. The functions of the thymus after thymectomy may be taken over to some extent by hormones from the thyroid (Ernström, U., and Gyllensten, L. [1965]. *Acta path. microbiol. scand.*, **64**, 193).

Studies of the action of thymic factors may assist our better understanding of the whole lymphopoietic process. Lymphopoiesis is not represented only by a number of successive mitoses —eight mitoses for the development of the small lymphocyte has been suggested by C. P. Leblond and G. Sainte-Marie (1960. *Ciba Fdn Symp. Haemopoiesis: Cell Production and its Regulation*, p. 152, London: Churchill)—but non-mitotic differentiation is also involved. The effect of the thymic factor may help to distinguish these two processes.

There is now good evidence that small lymphocytes can produce immunoglobulins and antibodies. It will be of interest to see if thymocytes can also do this. It has not yet been established if thymocytes can produce antibodies but these cells certainly can form immunoglobulins of non-antibody character. This has been shown in newborn piglets (Šterzl, J., *et al.* [1960]. *In* Mechanisms of Antibody Formation, p. 130, ed. Holub, M., and Jarošková, L. Prague: Czechoslovak Academy of Sciences), in rabbits (Humphrey, J. H. [1963]. *In* La Tolérance Acquise et la Tolérance Naturelle à l'Egard de Substances Antigéniques Définies, p. 401. Paris: CNRS) and in sheep foetuses stimulated with Freund's adjuvant (Silverstein, A.M., *et al.* [1963]. *J. Immun.*, **91**, 384).

The role of the thymus in resistance to infections and the effect of the thymus on immunity have been intensively studied. It is now well documented that the occurrence of post-thymectomy wasting disease is mainly due to micro-organisms and their products (Azar, H. A. [1964]. *Proc. Soc. exp. Biol. Med.*, **116**, 817; Brooke, M. S. [1964]. *J. exp. Med.* **120**, 375). Under germ-free conditions the wasting syndrome does not develop (Wilson, R., Sjodin, K., and Bealmear, M. [1964]. *Proc. Soc. exp. Biol. Med.*, **117**, 237). Although thymectomy was not found to have any influence on phagocytosis in experiments with colloidal carbon (Morrow, S. H., Di Luzio, N. R. [1965]. *Nature, Lond.* **205**, 193), little is known about the effect of the thymus in extreme conditions in which the phagocytic system has become exhausted

and regeneration is needed. It is also not known if thymectomy influences the formation of serum factors which possess opsonization activity, such as complement.

The influence of thymectomy on specific immunity, that is, on the delayed type of hypersensitivity reaction and on antibody formation, is well proved. Attention is consequently now stimulated by reports of situations in which antibody responses after thymectomy are not inhibited (Humphrey, J.H., Parrott, D.M.V., and East, J. [1964]. *J. Immun.*, **7**, 419). In view of results such as these, the hypothesis of the central function of the thymus in the development of immunity must be critically re-evaluated. This hypothesis suggests that cells from peripheral sources, probably from the bone marrow, immigrate to the thymus which functions as an educational centre for these cells; the immigrating cells acquire immunological competence in the thymus and are now known as immunologically competent cells or X cells (this volume, p. 36, foot-note); these cells then migrate to the peripheral lymphoid tissues. An alternative concept considers that the number of X cells in the peripheral lymphoid tissue is relatively constant and that the origin of these cells is not directly dependent on the thymus (Šterzl, J., and Mandel, L. [1966]. *Folia microbiol., Praha*, in press), but that the X cells are activated to cells with proliferative potential, Y cells, only after contact with the appropriate antigen. Under normal conditions the organism is antigenically stimulated during the first few days of life by the intestinal flora, since the diet contains most of the antigenic determinants which are later used as antigens for the development of immunity. The activated cells or Y cells proliferate and the thymic humoral factor plays an important role at this stage. In neonatally thymectomized animals, the proliferation of Y cells is depressed so that the response to injected antigen in these animals is impaired compared with intact controls. Our own experimental results so far are in agreement with this hypothesis. In germ-free sterile piglets the number of immunologically competent cells per 10^8 lym-

phoid cells is relatively constant throughout the growing period. Thymectomy does not affect this situation: control and neonatally thymectomized sterile piglets, at one month of age, have the same number of antibody-forming cells. This suggests that during growth the number of immunologically competent cells does not increase by passage of cells through the thymus. A difference in the number of antibody-forming cells in normal and thymectomized animals is only found if thymectomy is carried out in conventionally reared animals which have been in contact with many antigenic stimuli, or in sterile animals purposely immunized. In this situation thymectomy interferes with proliferation of the Y cells. This depression of the immune response by thymectomy may be similar to the effect of 6-mercaptopurine and other immunosuppressive drugs and may perhaps be used in the treatment of some of the immunopathological diseases in man.

Burnet: It might be useful now to discuss what new experimental work is needed in the field of immunology and the methods we should try to develop to pursue our studies. Three problems that I think are particularly suitable for discussion are (*a*) the mechanism by which cells leave the thymus and the bursa of Fabricius, (*b*) the development of simple, quantitative assay methods in immunology, and (*c*) the relationship of the thymus to immunological tolerance.

I know of no experiments that have specifically dealt with the lymphatic drainage of the thymus or the bursa and suggestions for methods by which this problem could be tackled would be most helpful. We have to deal with an apparent paradox: Dr. Metcalf has stressed there is no real evidence that any cell ever leaves the thymus and yet, functionally, we feel absolutely certain that quite a large number do.

Simple, accurate, quantitative methods for assaying the various hormones or humoral substances that may be secreted by the thymus, the bursa and perhaps as yet unidentified sources are

badly needed. There is strong circumstantial evidence that these substances exist and influence the viability and development of the lymphoid cells, but we have no direct proof for them yet.

Clark: Classical histological descriptions of the thymus agree that there are lymphatics in the interlobular connective tissue, but there is little knowledge or agreement about the presence of lymphatics within the thymic parenchyma itself (Bargmann, W. [1943]. *In* Handbuch der mikroskopischen Anatomie des Menschen, vol, 6, pt. 4, p. 1, ed. Möllendorff, W. von. Berlin: Springer-Verlag). Christiana Smith (1955. *Anat. Rec.*, **122**, 173) described tubular accumulations of densely packed lymphocytes arranged in a sleeve-like fashion around the major blood vessels in the thymus and called these accumulations lymphatics. In my studies with the electron microscope I have found no structures I could identify as lymphatics within the thymic parenchyma. The perivascular accumulations of lymphocytes described by Smith appear to lie in the connective tissue space between the wall of the blood vessel and the encircling layer of thymic epithelium.

In looking for other avenues by which cells may enter or leave the thymus, we should consider the venules. They are lined by high endothelial cells and lymphocytes can be seen in transit through this endothelium. Lymphocytes can also be seen between the epithelial cells that surround perivascular connective tissue where they occlude interruptions in the epithelial basement membrane. These observations suggest that lymphocytes penetrate the thymic-epithelial barrier and the walls of venules to pass between the thymic parenchyma and the bloodstream. Unfortunately, such static observations with the electron microscope do not allow us to determine the direction or extent of the migration of the lymphocytes.

Burnet: Many of our pathological thymuses show areas which we call "packed lymphatics". These are undoubtedly cavities choked with small lymphocytes. Dr. Clark, what do you think these appearances might indicate?

Clark: We see the same sort of thing, but to a lesser degree, in normal thymuses.

Burnet: And in normal lymph nodes?

Clark: Well, yes, but in the lymph nodes these collections of cells probably represent something different. In the normal thymus they occupy the perivascular connective-tissue spaces within the thymic parenchyma. The walls of the spaces consist of thymic epithelium and the spaces themselves can become enlarged and choked with lymphocytes.

Dameshek: There is no direct evidence that what I have called the immunoblast goes on to plasma-cell production with the appropriate stimulus. On the other hand, it seems clear that lymphocytes are produced from this large pyroninophilic blast cell.

About two centuries ago, William Hewson (1777. Experimental Inquiries: part the third. London: T. Longman) tried to discover the fate of the cells of the thymus and where they went. He never found a clear answer to these questions despite vigorous experimentation. He tied ligatures round the thymus and compressed the gland which produced an increase of lymphocytes in the thoracic duct. The work of two centuries has not advanced this particular cause very much.

Auerbach: There must be different approaches to the problem of cellular traffic in the mature animal (which includes the newborn by my definition!) and in the embryo. In the embryo a great deal of cell traffic needs no circulatory system; for example, the migration of germ cells into the gonad and of neural crest cells into the skin. When we look at the cellular traffic of the thymus in the prenatal period, therefore, we cannot look at it in the ways that Dr. Ford and Professor Gowans have described in their experiments with the mature gland. I wish I could tell you what approaches we should use. Perhaps we should be looking for capillary beds and lymphatic circulation in the mature animal, and for cell seeding in the embryo.

Ford: An experimental model which might be of value is the

artificial mosaic mouse. A. K. Tarkowski (1961. *Nature, Lond.*, **190,** 857) in Poland and Beatrice Mintz (1965. *Science*, **148,** 1232) in Philadelphia have both independently obtained such mice. In Tarkowski's method, two eggs at the δ-cell stage are fused together, cultured for 24–36 hours *in vitro*, and injected as blastocysts into the uteri of prepared females. Some of these blastocysts implant, grow to term and are born alive as "normal" mice. These mice are thus made up of two distinct kinds of cells that can be marked in any suitable way. I would naturally use a chromosome marker. Tarkowski has shown quite clearly that these mice really are mosaics; it is better to call them mosaics than chimaeras even though they are produced artificially.† These animals offer great possibilities for investigation as we can explore the ratio of the two sorts of cells in every tissue in the body. For example, if the thymic rudiment arises from a very few cells its initial constitution may be all of one type of cell. If this is so, we may be able to learn something of the relationship of the thymus with the other organs of the lymphoid system.

Furth: It seems likely that lymphocytes leave the thymus by the process of emperipolesis (Ioachim, H., and Furth, J. [1964]. *J. natn. Cancer Inst.*, **32,** 339). Alice Moore has shown with motion pictures that lymphocytes can pass through epithelial-reticular cells, and even through their nuclei, without injuring cells or nuclei.

Clark: But this was in lymph nodes, not in the thymus.

Metcalf: Cell migration from the thymus in the venous blood has been postulated by G. Sainte-Marie and C. P. Leblond (1962. *In* The Thymus in Immunobiology, p. 207, *loc. cit.*). These workers counted lymphocytes in sections of thymic arteries and veins in

† The two terms have been variously used in the literature. Both imply individuals compounded of two or more genetic or karyotypic classes of cells. On the whole mosaic places emphasis on natural origin early in embryogenesis, whereas chimaera relates to events after embryogenesis, for example, the natural exchange of cells between twin foetuses by cross-circulation, or artificial introduction of cells from another animal.

the rat. Another way of tackling this is by cannulating the arterial and venous blood supply to the thymus in a large animal, such as the puppy, and counting the cells that pass in and out of the major vessels. This is technically feasible and a technique is currently being developed by Dr. M. Symonds of the Royal Children's Hospital, Melbourne.

Nossal: We have all been avoiding the very important issue of the sequence of events in the life of the lymphocyte. We have accepted C. E. Ford's concept that a cell from the bone marrow can enter the thymus and subsequently be seeded out to peripheral lymphoid tissue, but we have not discussed fully whether we all accept R. Auerbach's concept that these lymphoid cells originate in the epithelium of the thymus or other parts of the pharyngeal pouch and then migrate to the bone marrow. The sequence of events might be something like this: the thymic epithelial cell generates the thymocyte which migrates to the bone marrow before birth and becomes the vital stem cell for the rest of life; the generation of such cells from thymic epithelium may cease shortly after birth; the sequence for the lymphocyte may therefore be: thymus to bone marrow to thymus to lymph node.

Ford: I think this sequence is entirely possible; it is certainly not excluded by any evidence that we have so far. The results I presented on the transplantation of bone-marrow cells from parabionts into irradiated hosts (this volume, p. 141) suggest that there is a differentiated compartment of cells in the bone marrow. The cells in this compartment have been separated from other cells by their mobility in the parabiotic mice and identified by their capacity to move preferentially to lymph nodes in the new host. Other properties of these cells will be interesting. It should be possible to devise a formal experiment to test if they come initially from the thymus or if they originate in the bone marrow with the other bone-marrow cells. What do the embryologists think about the origin of these cells, Dr. Auerbach? The first myeloid tissue appears in the yolk sac, doesn't it?

Auerbach: First of all, I am not sure I accept what Dr. Nossal refers to as "Auerbach's concept of the origin of lymphoid cells"!

The first question that we have to answer is this: is there a single origin for all the lymphoid cells or do they have multiple origins? If they have a single origin, and since we can show that some of them come from the thymus, then the only origin for the lymphoid cells must be the thymus. But other cells in the body have multiple origins, for example the blood cells. In the embryo, blood cells derived from the yolk sac are quite different from blood cells derived from the liver and these again are different from blood cells derived from the spleen. It is not possible to confirm or refute Dr. Nossal's sequence until we know if lymphoid cells have a single or a multiple origin.

Good: A look at this problem from the phylogenetic angle may be useful. Long before the thymus develops, living organisms contain cells that look like lymphocytes. We must decide what the lymphocyte that we are talking about really is. Are there many different types of lymphocyte, or one type, or lymphocytes that look alike but are in various stages of differentiation? We have devised nine different parameters in an attempt to define the different forms of lymphocytes (this volume, p. 198, table II), and we will use these parameters to study the lymphocytes of primitive creatures. D. Thomas (1965, *J. Lab. clin. Med.*, **65**, 794), in some very exciting work on the differential effects of alkylating agents and X-rays, has demonstrated what appears to be a small lymphocyte that is a source of granulocytes and red blood cells in the bone marrow of the dog. I think the lymphocyte that immigrates into the thymus may not be the same type of lymphocyte that, as Auerbach has shown, arises in the thymus, but it may ultimately be able to differentiate into this latter sort of lymphocyte. We regard the lymphocyte that comes from the bone marrow as a lymphoid haemoblast.

Feldman: The origin of lymphocytes in relation to the thymus has been studied in three different systems: the embryo, the new-

born and the adult. Dr. Auerbach has demonstrated that thymic lymphocytes in the embryo develop from the epithelial components of the thymus. The *in vitro* systems for antibody formation and for graft-versus-host assay recently developed in Dr. Auerbach's laboratory, may be applied to answer the question of whether lymphocytes that originate in the thymic epithelium migrate out of the thymus and become immunologically competent.

After total-body irradiation of the adult organism cells in the bone marrow have been shown by Dr. Auerbach's team to be the precursors of lymphoid cells. Lymphocytes originating in the bone marrow seem able to differentiate into immunologically competent cells under the inductive influence of the thymus. But we do not know if the bone marrow under *normal* conditions, that is, without X-irradiation, is the reservoir of cell primordia for lymphoid differentiation. The fate of isotopically labelled bone-marrow cells introduced into non-irradiated, normal hosts would be one way of studying this problem.

From our observations that (*a*) the thymus instructs bone-marrow-originated cells to differentiate into lymphoid cells, and (*b*) this inductive effect can be mediated by thymuses implanted within diffusion chambers, it has been assumed that the thymus functions by means of a hormone. Talking about thymic hormones has already led to some confusion. A typical hormone is a substance which is secreted by a gland and transported in the blood stream to act on a target organ anatomically distinct from the secreting gland. So far there is no evidence that the thymus *under normal conditions* does secrete any such substance. If bone-marrow cells do migrate into the thymus to be "instructed" there, it suggests an intrathymic induction by cell contact interaction rather than a hormonal effect; morphogenetic inductions during embryogenesis seem to function by such cell-contact interactions. The fact that thymic tissue also "induces" when it is implanted in a diffusion chamber may be due to the effects of the

cytolysis which takes place within the chambers during which the inducing substance may leak out. The way in which the thymus functions—as a hormone-secreting organ or by local cell-contact interaction between the epithelial component and other cells—remains a subject for further investigation.

Metcalf: I am not convinced that the epithelial primordia that Dr. Auerbach removes are a homogeneous population. These cells may all look the same but this does not necessarily mean that they are the same. One of the disturbing facts about thymus repopulation in the adult mouse is that the repopulating cells move into the thymus through the medulla. It seems possible that the first primordial lymphoid cells in the embryonic state may also enter the thymus through the medulla and thus, when a so-called epithelial primordium is removed by Dr. Auerbach, it may already contain contaminating primitive lymphoid cells. These primitive cells look very similar to primitive epithelial cells.

We have used another system which avoids destruction of the thymus and the use of irradiation to study the repopulation of the thymus. $(AKR \times C3H)F_1$ hybrid mice are injected with AKR cells from various organs. The marker we use to identify migrant AKR cells in the thymus is the development, some months later, of lymphomas in the thymus. Test transplantation of these lymphomas will reveal which tumours are derived from the injected AKR cells. We have shown with this system that when normal AKR spleen cells are injected intraperitoneally into normal young adult $(AKR \times C3H)F_1$ animals at least some of the cells migrate to the thymus. They persist and proliferate there, and ultimately, after many months, develop into lymphomas (Metcalf, D. [1962]. *Ciba Fdn Symp. Tumour Viruses of Murine Origin*, p. 233. London: Churchill). This same system could be used to determine if cells from other tissues migrate to the thymus.

Gowans: There are considerable technical difficulties in studying the export of cells from the thymus. The interpretation of anatomical studies alone is full of pitfalls. Photographs showing cells

in transit through the walls of blood vessels can give no information about the direction of migration, nor about the magnitude of the process. An ideal approach would be to perfuse the whole thymus with tritiated thymidine continuously in order to ensure uniform labelling of all the dividing cells. J. G. Hall and B. Morris (1965. *J. exp. Med.*, **121**, 901) have achieved this in the lymph nodes of sheep by infusing tritiated thymidine slowly into afferent lymphatics, but unfortunately these do not exist in the thymus. I was wondering if the same end might be achieved by a very slow arterial infusion close to the thymus, with safeguards to ensure that tissues elsewhere did not become labelled by excess radioactive material leaving the thymus. This complication might be minimized by the continuous infusion of a high concentration of non-radioactive thymidine at another site in the animal, as in the experiments of Irving Weissman (unpublished material) which Dr. Kaplan described (this volume, p. 267).

Nossal: Judith Mitchell and I thought about doing this sort of experiment in sheep. We discussed the problems of arterial and venous transplantation techniques with Dr. D. Denton of the Howard Florey Laboratories. We had hoped to transplant the thymus, with its arterial and venous supply intact, to an alternative site such as a fold in the neck and then do our physiological experiments with labelled cells. Unfortunately, there are no colonies of inbred sheep and Denton advised us that the technical problems of this sort of experiment were so great that it was not worth trying. If the sheep ever reached the operating table the blood supply of the transplanted thymus would make it extremely difficult to do a clean experiment and keep the animal alive. We really need a large animal that is inbred for this work.

Gowans: I agree that the transplantation experiment would be very difficult in a small animal, but a continuous slow arterial infusion into an unrestrained rodent is perfectly feasible.

Nossal: The perfusing blood needs to be continuously monitored for everything including its hormonal content.

Gowans: You are being too ambitious. Useful information could be obtained by examining the peripheral lymphoid tissue after various periods of continuous infusion in different individual animals.

Kaplan: There is a remarkable and exquisitely sensitive interaction between the thymus and many of the endocrine glands (Kaplan, H. G., Nagareda, C. S., and Brown, M. B. [1954]. *Recent Prog. Horm. Res.*, **10**, 293). Certain components of the pituitary as well as the adrenal secretory system, the thyroid and the gonads are probably involved in it. This must surely, from a teleological standpoint, have an important function in the defence mechanisms of the host. These interactions have not been re-explored in the light of modern knowledge of the role of the thymus. We know nothing about the action of corticosteroids on the epithelial components of the thymus. What, for example, would be the effect of adding corticosteroids to Dr. Auerbach's embryonic culture-systems at selected stages? It would be interesting to see the results of endocrine manipulations on some of the secretory manifestations in the thymus demonstrated by Dr. Clark. A return to the endocrine system might lead to an elaboration of new clues which would help unravel the mechanisms behind some of the functional interrelationships and responses with which the thymus is connected.

Auerbach: These studies are in progress.

Furth: Growth hormone secreted by the acidophilic cells of the anterior pituitary gland causes a marked enlargement of the thymus (Takemoto, H., *et al.* [1962]. *Cancer Res.*, **22**, 917). The simplest assay for growth hormone is, in fact, thymic weight, provided the adrenal has not been stimulated, for growth hormone is strongly antagonized by ACTH and corticosteroids, both of which cause thymic involution. To eliminate this antagonism the assays are best done in adrenalectomized animals. Decrease in thymic weight is a quantitative indicator of adrenal-mediated stress.

Animals bearing thyrotropic hormone are said to have an in-

creased incidence of lymphoid neoplasms (Sproul, E. E., and Grinberg, R. [1964]. *Cancer Res.*, **24**, 1644) but these are not thymic lymphomas. Lymphoid stimulation in human hyperthyroidism is well known.

Metcalf: Avian immunologists (Warner, N. L., and Szenberg A. [1964]. *In* The Thymus in Immunobiology, p. 395, *loc. cit.*) have shown that large doses of testosterone inhibit the development of the thymus as well as the bursa. Testosterone affects the epithelial-cell elements of the thymus and it would be most valuable to examine this change in detail by electron microscopy.

Boyden: An important problem in relation to the thymus and immune responsiveness concerns the method by which the antibody-forming system is enabled to recognize antigens. Although the theory of clonal selection suggests an explanation for this ability to discriminate between antigens this theory is not yet proven. This mechanism of recognition is an extremely important part of the immunological system and we still do not know if it is controlled from within the thymus or from outside it. We do not know if lymphocytes possess the capacity for discrimination initially, if it is conferred on them during development outside the thymus, or if other elements—for example, macrophages or even humoral elements—are involved. The following experiment would help to elucidate this problem: it has been shown that immunological unresponsiveness after the injection of antigen into neonatal animals can be overcome with syngeneic spleen cells. Can this unresponsiveness also be overcome by thymus grafts? The answer to this question would surely provide us with a clue to one of the most important problems of antibody theory.

Metcalf: B. F. Argyris (1962. *Transplantatn Bull.*, **30**, 140) has studied the breakdown of acquired tolerance to skin homografts in mice. Tolerance breaks down slowly with the passage of time, but the rate of tolerance breakdown can be accelerated by thymus grafts and thymus grafts in diffusion chambers (Argyris, B. F. [1965]. *Transplantation*, **3**, 350). In the latter situation a thymic humoral factor is either conferring new reactivity patterns on cells

or accelerating the rate at which cells with these patterns proliferate when the animal is challenged.

Good: It is the rate of tolerance breakdown that is important here.

Metcalf: I agree; the influence of the thymus is probably a rate-accelerating one.

Has anyone tried the effect of thymic extracts on immunologically competent cells *in vitro*? Such a procedure might be applicable to the Jerne plaque technique. It should be simple to find out if a layer of agar containing thymic extract allowed more plaques to develop than agar alone. Would it be feasible to keep these cultures for long enough to allow *in vitro* proliferation? Has anyone any experience with this technique?

Feldman: There might be one restriction here. E. Lennox has told me (personal communication) that the formation of plaques by mouse cells is due to the release of preformed antibody (that is, antibody formed *in vivo*), and is not a reflection of antibody synthesis *in vitro*. The inhibition *in vitro* of protein synthesis by puromycin does not inhibit the formation of plaques by cells which have been pre-immunized by sheep red blood cells. The new system for the *in vitro* production of antibody developed by Globerson and Auerbach may be of use here (this volume, p. 39).

Good: Are we completely condemning the plaque technique?

Metcalf: Only as a method for estimating antibody *production* by single cells *in vitro*. The technique measures only the release of preformed antibody.

Miller: Dr. Metcalf, have you been able to repeat your early work with the lymphocytosis stimulating factor? Have you confirmed that thymus extracts stimulate lymphocytosis in thymectomized or in intact mice?

Metcalf: In the intact adult mouse we have never observed lymphocytosis, an increase in lymph node weight or a change in the thymic mitotic indices or thymic weight after injections of thymic extract from syngeneic mice. The only type of animal in which

we did find some of these changes was the irradiated, thymectomized, adult mouse (Metcalf, D. [1959]. *Radiat. Res.*, **10**, 313). There are reports in the literature on the positive effect of thymic extracts on many of these parameters but we have not found this. J. J. Klein, A. L. Goldstein and A. White (1965. *Proc. natn. Acad. Sci. U.S.A.*, **53**, 812) have claimed that a single injected dose of calf, rat or syngeneic mouse thymic extract increased the uptake of tritiated thymidine in the lymph node cells of the adult mouse. These workers found that an injection of this material daily for three days increased lymph node weight and [^{14}C] glycine uptake by lymph node cells. We are at present trying to repeat these results. Klein, Goldstein and White have found that thymic extracts prepared at pH 5·7 were active whereas extracts prepared at pH 7·4 were not. A. Nakomoto (1957. *Acta haemat. jap.*, **20**, 187) has also reported that acid thymic extracts were active. In our experiments with thymic extracts we may not be using the right medium for extracting adequate titres of lymphocytosis stimulating factor or we may be extracting inhibitors as well as stimulators or doing our extraction procedures at an inappropriate pH. I have no idea why a pH of 5·7 should be so much better than a pH of 7·4; it might be worth following this up.

Good: Dr. Metcalf, the donor of the lymphocytosis stimulating factor in your experiments was of a different species from the recipient mouse. Our attempts to stimulate lymphopoiesis by single and multiple transplants of rat thymus into the neonatally thymectomized mouse have also been unsuccessful. We have had great difficulty in reconstituting the *mouse* with *rat* thymus but have been more successful when we used thymus grafts from mice. This suggests that we should remain within one species when we work with these extracts. The calf and whale extracts suggested by Szent-Györgyi and others (Szent-Györgyi, A., Hegyeli, A., and McLaughlin, J. A. [1962]. *Proc. natn. Acad. Sci. U.S.A.*, **48**, 1439), in spite of their availability in vast quantities, would probably be less useful for mice than mouse extract.

Metcalf: We have been able to demonstrate the presence of lymphocytosis stimulating factor in mouse thymic tissue when this has been tested in syngeneic baby mice, but we have also found activity in human thymic extracts on our mouse assay-system. The use of xenogeneic or even allogeneic preparations of thymic extracts worries me too. This is why I was so interested in Klein, Goldstein and White's report (1965, *loc. cit.*). Although they found a higher activity with calf and rat thymic extracts, they were able to show activity with syngeneic mouse extracts too. There are many problems when a foreign (potentially) antigenic source of thymic extract is used.

Good: Dr. Miller, has Dr. Law been successful in reconstituting his animals with foreign thymus grafts?

Miller: Yes. He has grafted thymus tissue from newborn rats (Osborn-Mendel) or hamsters (*Mesocricetus auratus*) into neonatally thymectomized (C57BL × C3Hf)F_1 hybrid mice within a day of thymectomy. From 60 to 70 per cent of the mice were restored in their capacity to react immunologically, even though regeneration of the implants to typical thymic structure was not observed. Epithelial-reticular cells persisted in the graft for about 30 days, but eventually polymorphonuclear-cell invasion took place and central necrosis and fibrosis followed. No grafts could be recovered from any of the F_1 mice killed between three and seven months (Law, L. W. [1966]. *Nature, Lond.*, in press).

Burnet: How did Dr. Law measure this reconstitution?

Miller: He used several immunological parameters including the rejection of AKR skin grafts and the production of haemolysins and haemagglutinins against sheep erythrocytes. About 80 per cent of thymectomized controls retained AKR grafts for more than twenty days and most had intact skin grafts when they died of wasting disease at two to five months. More than 60 per cent of thymectomized mice bearing xenogeneic thymic implants rejected AKR skin within twenty days. Haemolysin production was low in thymectomized controls but in the thymectomized,

thymus-grafted mice it was within the range of the sham-operated animals. Dr. Law's results fully agree with those reported by our group with allogeneic thymus grafts: C57BL thymuses restored immunological capacity in thymectomized irradiated CBA mice even though the implants themselves were eventually rejected (Miller, J. F. A. P., *et al.* [1964]. *Ann. N.Y. Acad. Sci.*, **120**, 205; Dukor, P., *et al.* [1965]. *Transplantation*, **3**, 639).

Auerbach: In our search for reliable assay systems we need a series of good reproducible extracts and a good test system. A simple and suitable test system might be irradiated spleen *in vitro*. A. Globerson, L. Fiore-Donati, and M. Feldman (1962. *Expl Cell Res.*, **28**, 455) have shown that restitution of the spleen requires the thymus, and D. Osoba and J. F. A. P. Miller (1963. *Nature, Lond.*, **199**, 653) demonstrated, by marker experiments, that this restitution is due to indirect activity of the thymus. An irradiated spleen will not regenerate without this stimulus. In this simple system we could add known amounts of different thymic extracts and measure *in vitro* the lymphoid changes that arise in the spleen.

Feldman: What is the relationship between the lymphopoietic effect of the thymus and its action in inducing immunological competence? Dr. Miller has claimed that his experiments with thymuses exposed to either 500 or 2000 r. indicate that the thymus functions through two distinct factors: one of these stimulates lymphopoiesis and the other confers immunological competence. If the transplantation of heavily irradiated thymuses to thymectomized animals had resulted in lymphoid regeneration in the spleen and lymph nodes without the manifestations of immunological competence, Dr. Miller's conclusion regarding the two functions of the thymus would have been justified. But what actually happened was that the spleen and lymph nodes of animals grafted with irradiated thymuses did not show lymphoid regeneration, only the implanted thymuses themselves showed this restoration. No experiments were made to test whether these

"restored" thymocytes were immunologically deficient compared with the thymocytes of normal thymuses. This could be studied by testing the capacity of the thymocytes to elicit a graft-versus-host response. We should postpone our talking on the heterogeneity of thymic hormones until this work has been done.

Miller: I agree that the only tests we applied were on the whole animal to see if it could produce plaque-forming cells or reject skin grafts. We shall, as you have suggested, now use cells from irradiated thymus grafts to test their capacity to produce a graft-versus-host reaction. However, the lymphoid tissues of thymectomized animals grafted with thymic implants irradiated with 2000 r. were, in most cases, indistinguishable morphologically from the lymphoid tissues of thymectomized controls grafted with normal thymus tissue. The blood lymphocyte levels were also comparable, yet only the mice grafted with heavily irradiated thymuses had poor immunological responses. In other experiments (Osoba, D., and Miller, J. F. A. P. [1964]. *J. exp. Med.*, **119**, 177) 7-day-old, neonatally thymectomized mice were implanted intraperitoneally with Millipore diffusion chambers containing either 14-day-old embryonic, or neonatal, thymus tissue. These mice were able, at the age of five to six weeks, to reject homografts and produce antibodies to sheep erythrocytes. These phenomena occurred in spite of the fact that the majority of these mice had subnormal lymphocyte levels in the blood and in most lymphoid organs. This evidence suggests, therefore, that it is not the quantity of lymphocytes present, but rather their immunological competence which is the critical factor in determining whether mice display normal immune responses or not.

Furth: In vivo assays of the presumed thymic hormones present a problem. The protein hormones usually used in such assays are derived from xenogeneic species. Thus, although the hormones are cross-reactive as hormones, they are also antigenic and can only be used for short-term assays.

Auerbach: The problem of antigenicity in test extracts is a very

real one. Even syngeneic extracts may become antigenic because the method of their extraction—their actual chemical treatment —may make an otherwise perfectly inert compound antigenic. The result of this antigenic change is to stimulate lymphopoiesis which invalidates the results of the experiments.

Metcalf: As a control for the non-specific production of antigenic substances during their chemical extraction it is probably adequate to use similarly prepared extracts of spleen and lymph node from the same donor.

Auerbach: I do not agree. If we can develop a reaction to our own antigens, we can certainly be stimulated to react to the presence of chemically produced foreign antigens in our blood.

Metcalf: Yes, but thymic extract experiments are always controlled by using extracts from a number of different tissues. It is surely a little unreasonable to expect that a chemical treatment will make thymus tissue develop one sort of antigen and lymph node tissue another.

Miller: Dr. Metcalf, would you tell us on which type of cell you think the lymphocytosis stimulating factor acts? Is it X, Y or Z cell, according to Dr. Šterzl's classification? (this volume, p. 35, foot-note).

Metcalf: First of all, I am not absolutely clear about terminology. Dr. Šterzl, why do you say that a specific reactivity pattern develops in the Y cells but not in the X cells?

Šterzl: The specific pattern may already be present in the immunologically competent cells, the X cells, although it is not expressed. Contact with an antigen induces the expression of this competence with a simultaneous change in the cells. That is, the X cells differentiate into immunologically activated Y cells which themselves possess the ability to proliferate.

Metcalf: The evidence we have suggests that the lymphocytosis stimulating factor does not operate before the stage of the X cell. Sir Macfarlane has postulated that the thymus might generate lymphoid cells with new reactivity patterns (X cells) on the

basis of random mutations (Burnet, F. M. [1962]. *Br. med. J.*, **2**, 807). We have studied very old C57BL mice in which immune responses are depleted. T. Makinodan and W. I. Peterson (1964. *J. Immun.*, **93**, 886) have shown that such mice contain reduced numbers of cells capable of being stimulated by antigens, although the cells that are present can be stimulated. In other words, the X cells or the Y cells are depleted in these animals. We grafted these old C57BL mice with massive amounts of neonatal C57BL thymus tissue and after two or three months we tested them for immunological competence: we found that we had not improved their immune response to sheep red blood cells (Metcalf, D. Unpublished data). There are many reasons why this experiment may have failed (for example, deficient antigen trapping and processing by the macrophages) but the result does suggest that the thymus cannot be the only, or even the main, tissue generating lymphoid cells with new reactivity patterns.

Gowans: Why did you not give the animals bone marrow as well as thymus?

Metcalf: Because we were trying to find out if the thymus, unaided, could create new cells that could react with the antigens of sheep red blood cells. If we had wanted to find out whether the thymus could expand a population of cells from the bone marrow that already had this reactivity pattern, then we would have given bone marrow as well as thymus.

Gowans: Have you done any studies in these old mice with a combination of thymic extract and bone marrow cells?

Metcalf: Not yet. Bone marrow alone does not restore full immunological competence. We plan to test the combination of thymus and bone marrow quite soon.

Gowans: Dr. Metcalf, could you summarize your ideas about thymic hormones? Does present evidence force us to conclude that there are two separate thymic hormones or just one?

Metcalf: At this stage it is probably not necessary to postulate the existence of two or more hormones. I think that the thymic

hormone acts, in association with antigen, on antigenically competent cells to accelerate their proliferative response after antigenic stimulation. H. Friedman (1965. *Proc. Soc. exp. Biol. Med.*, **118**, 1176) has shown that only a low degree of proliferation is possible in the absence of thymic hormone. I am really not sure if the accelerated proliferation of the plasma-cell and lymphocyte series results from the catalytic action of a single thymic humoral factor, or if two separate hormones are needed for these two actions. I do not understand why antigenic stimulation for lymphopoiesis does not seem to be needed in the thymus itself. Here the humoral factor—presuming it is the same factor in the thymus as in the lymph nodes—can stimulate lymphopoiesis alone, without stimulating antibody production. Is there a second factor for the thymus or is it just that two basically different cell types respond to the same humoral factor in different ways? With the evidence available we can probably postulate that there needs to be only one hormone, provided we also assume that the dual response to antigenic stimulation—lymphopoiesis and plaque-forming cell (plasma cell) proliferation—is regulated by a single factor.

Gowans: Does this mean that you think the action of this hormone is to induce proliferation of cells rather than specific immunological reactivity?

Metcalf: Yes. The evidence indicates that the thymus does not dictate reactivity patterns.

Clark: Dr. Metcalf, why do you think the thymic hormone has to interact with antigen? Is it not possible that the hormone only stimulates proliferation of the cells, and the apparently heightened response to antigen is simply a consequence of more cells being available to respond?

Metcalf: The evidence against this is that the germ-free animal has very little lymph node tissue but a fully-developed thymus (Gordon, H. A. [1959]. *Ann. N.Y. Acad. Sci.*, **78**, 208). In germ-free animals the blood lymphocyte levels also are subnormal.

This suggests that the production of lymphocytes in lymph nodes and blood needs antigenic triggering as well as thymic stimulation.

Clark: I am supposing that the thymic hormone can promote proliferation not only of uncommitted cells but also of previously committed cells and, therefore, that it may play a role in maintaining the number of previously committed cells in the absence of antigen.

Metcalf: This is possible.

Gowans: In other words, the hormone should have an effect on the magnitude of the secondary response.

Good: G. J. Thorbecke and co-workers (1957. *J. infect. Dis.*, **101**, 237) showed, although there has been some argument about this, that the bursa in the germ-free chicken is normal while the rest of the lymphoid tissue is poorly developed. Dr. Miller, what are the tonsils and Peyer's patches like in the germ-free animal?

Miller: I do not know.

Šterzl: Germ-free animals fed with a non-antigenic diet showed no significant increase in the proportion of immunologically competent cells per population of 10^8 lymphoid cells during the first months of life; immunological competence was assessed by the number of cells which produced antibodies to sheep red blood cells. The number of competent cells does not increase if the germ-free animals are stimulated non-specifically by the injection of lipopolysaccharide or Freund's adjuvant. Therefore, I would not expect the proliferative activity of immunologically competent cells to increase but only the activity of the activated Y cells. I would also expect the influence of inhibitory and stimulating substances, such as the postulated thymic factor, to act on the compartment of immunologically activated cells.

Good: Dr. Šterzl, did you look at the tonsil in these animals?

Šterzl: No.

Kaplan: Dr. Šterzl has suggested (this volume, p. 497) that wasting in neonatally thymectomized animals is due to bacterial in-

fection. I am not sure that I agree with him. We searched for bacteria preterminally in (C57BL × BALB/C)F_1 hybrid mice with wasting disease and were impressed to find that almost all the dying animals were bacteriologically sterile (Kaplan, H. S., and Rosston, B. H. [1959]. *Stanford med. Bull.*, **17**, 77). Thus, in our experience, wasting associated with the graft-versus-host reaction need not involve bacterial infection. The role of bacteria in conventional animals might be to introduce not infection as such but exogenous antigens. These antigens, in some way that we do not understand, might initiate a type of atrophic reaction similar to what is seen in the experimental graft-versus-host situation. Dr. Šterzl, do you think that this is possible?

Šterzl: The mechanism for the production of wasting disease in neonatally thymectomized animals is evidently different from the mechanism that produces runting through transplanted cells in the graft-versus-host reaction. I can only repeat that I believe, after our recent studies, that the post-thymectomy wasting syndrome is caused by micro-organisms or their products. Under germ-free conditions we did not see the wasting syndrome. I agree with Dr. Kaplan that in the preterminal stages in these wasted animals actual bacteriaemia is not demonstrated and so the harmful effect of the bacteria may be caused by endotoxins.

CHAIRMAN'S CLOSING REMARKS

Burnet: As Chairman of this symposium, I have the privilege of having the last word! It has been a pleasure to hold this meeting in Melbourne after many of us have taken part in similar conferences in other parts of the world.

I would like to try to sum up what has emerged from our discussions about the functions of the thymus in such a way that our deliberations will be useful to any teacher in the general medical sciences. This will involve dogmatic statements and I am sure that some of you will disagree with almost everything that I say. I hope though, that most of what I say will be broadly acceptable as a simplified overall picture of what has emerged from our discussions.

The suggestions that we have made are:

(*a*) The thymus is the centre of lymphoid-cell differentiation. In other words, multipotential, undifferentiated cells from the bone marrow, and possibly elsewhere, are differentiated in the thymus into immunocytes in which the genetic loci associated with antibody synthesis have been activated.

(*b*) At least 95 per cent of the cells produced in the thymus are destroyed there. I think myself that this may be because only a small proportion of the immune patterns that emerge with differentiation are acceptable to the body.

(*c*) Lymphoid cells emerge from the thymus and migrate to the second-level lymphoid organs. This outflow of cells from the thymus represents the main or only source for the introduction of new immune patterns into the organism. These patterns are thereafter conserved by replication and/or by the persistence of a small proportion of lymphocytic memory cells.

(*d*) Plasma cells are derived from lymphocytes through antigenic, or some equivalent, stimulation. The provision of a suitable internal environment for the plasma cells is of great importance, and hormonal factors may be concerned with providing this environment.

(*e*) Several hormonal factors are associated with the thymus and probably also with whatever bursa-like organs are present in the mammal. These hormones are responsible for primary lymphopoiesis and there may be at least three thymic hormones. The first of these is probably produced by the epithelial-reticular cells of the thymus and regulates lymphopoiesis in the thymus itself and perhaps also in the bursa-like organs. The second hormone may be necessary to maintain the initial viability of the immunocytes which leave the thymus and colonize in the secondary lymphoid organs. This hormone arises in the thymic epithelial-reticular cells and is the standard humoral agent most discussed in experimental studies. The third hormone is needed if immunocytes are to become plasma cells freely synthesizing immunoglobulins. This is presumably produced by gut-associated, epithelial-lymphoid organs which may or may not also serve as subsidiary centres of primary differentiation of immunocytes.

Most of what I have said is still hypothetical, but it does represent a consensus of the working pattern that has emerged from this symposium.

Finally, I would like to suggest three possible approaches which should be useful for our future research.

First, we must develop a fully professional attitude to the isolation, characterization and assay of the thymic hormones. We need better assay methods and better ways of extracting these substances in substantial quantities. Extraction may be accomplished, as Professor Furth suggested, by using material from specifically-active tumour cells, but we must devise other ways of dealing with these very important problems.

Second, work on the phylogeny of the immune response must

be extended and elaborated in line with developing knowledge of the actual physiological processes concerned. This line of research has been pioneered by Professor Good and I hope that our studies in Australia on the marsupials and the monotremes may fill up some of the evolutionary gaps.

My final suggestion is that we should continue to search for genetic variants, particularly different types of naturally occurring runts, in our mouse colonies. The NZB mouse and its hybrids still represent a fruitful potential source of knowledge of auto-immune processes. It was not too difficult to produce the NZB strain and there may be large untapped sources of genetic variants that would be extremely useful for immunological study. A spontaneously occurring situation, genetically determined, such as natural runting must be much more akin to the immune situation we have to deal with in real life than laboratory-made artefacts produced by grossly unphysiological procedures on normal animals.

I hope it has been useful to make these observations; they are mainly to help people who are not working specifically on the growing edge of thymus research to understand what we are thinking about the thymus at the present time.

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