Intestinal Immunity. The role of intraepithelial lymphocytes.

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Publications

The following publications contain work described in this Thesis:

Toner P G, A Ferguson (1971)

Intraepithelial cells in the human intestinal mucosa.

Journal of Ultrastructure Research 34, 329

Ferguson A, D Murray (1971)

Quantitation of intraepithelial lymphocytes in human jejunum.

Gut 12, 988

Ferguson A, D M V Parrott (1972)

Growth and development of "antigen-free" grafts of foetal mouse intestine.

Journal of Pathology 106, 95

Ferguson A, D M V Parrott (1972)

The effect of antigen deprivation on thymus-dependent and thymus-independent lymphocytes in the small intestine of the mouse.

Clinical and Experimental Immunology 12, 477

Parrott D M V, A Ferguson (1973)

Selective migration of lymphocytes within the mouse small intestine

Immunology (in press)

Ferguson A, D M V Parrott (1973)

Histopathology and time-course of rejection of allografts of mouse small intestine

Transplantation 15, 546

Abbreviations

B mouse	- thymectomised, irradiated, bone marrow reconstituted mouse
B lymphocyte	- bone marrow derived, thymus-independent lymphocyte
DNA	- deoxyribonucleic acid
H & E	- haematoxylin and eosin
IE	- intraepithelial
Ig	- Immunoglobulin
LN	- lymph node
MGP	- methyl green pyronin
N mouse	- normal mouse (as contrasted with B mouse)
NA	- nodular area
P	- probability
RNA	- ribonucleic acid
SD	- standard deviation
SE	- standard error of the mean
SI	- small intestine
TDA	- thymus-dependent area
T lymphocyte	- thymus derived lymphocyte

Chapter 1

Introduction

The villi and crypts of the small intestine are lined by a columnar epithelium of absorptive cells, the enterocytes. This orderly epithelium is interrupted by the presence within it of many non-epithelial cells, the majority of which are lymphocytes. Indeed, of the cells covering the enormous surface area of the villi, at least one in ten is a lymphocyte. These small intestinal intraepithelial lymphocytes form the subject of this thesis.

When I began this work, more than a century after Eberth's original description of these cells in 1864, there were several features of the intraepithelial lymphocytes which were undescribed, disputed or inadequately investigated (discussed in Chapter 2). There were many theories as to their origins and fate; their function was unknown; the nature of their relationship with adjacent enterocytes was disputed. I approached the investigation of these intraepithelial (IE) lymphocytes by attempting to answer the following questions:

What is the morphological relationship between the IE lymphocytes and adjacent non-lymphoid tissues?

Do IE lymphocytes form a homogeneous group of cells, or can thymus-dependent and thymus-independent populations be identified; are there populations specifically related to the presence/

presence of antigens within the gut?

How do the IE lymphocytes resemble or differ from the other lymphocytes of the small intestine (e g Peyer's patch lymphocytes)?

Is there any evidence that IE lymphocytes are altered in numbers, morphology or function in intestinal and immunological diseases?

These problems have been approached in two ways - by morphological examination and quantitation of human jejunal IE lymphocytes, and by a series of experiments in mice. A new experimental model was devised for the animal work. This provided "antigen-free" grafts of small intestine, and allowed investigation of the effects of intraluminal antigen deprivation on the small intestinal lymphoid tissues, the factors which influence migration of lymphocytes to the small intestine, and the morphology of allograft rejection of small intestine.

Human jejunal intraepithelial lymphocytes

Biopsy of the upper small intestine is now a routine part of the clinical investigation of patients who may have disease of the small intestine. These biopsies provide an excellent source of material for the study of human jejunal IE lymphocytes in health and in disease. A series of jejunal biopsies, 250 in all, were examined by light or electron microscopy, and numbers of IE lymphocytes were quantitated in 200. It proved possible to establish a normal range of values for jejunal IE lymphocyte counts, so that the influence of intestinal and immunological diseases on their numbers could be firmly established. Special emphasis was paid to the IE lymphocytes in patients with coeliac disease, where the jejunum is intensely inflamed and there is evidence of intolerance, perhaps allergy, to some cereals (Booth and Dowling, 1970).

An experimental model for animal work

The biology of the immune response has been extensively studied in rodents and chickens, and the availability of inbred strains of rats and mice makes these species especially suitable for research in cellular immunology. However, a major problem arises when one attempts to design experiments on intestinal lymphocytes in these small animals. Large

numbers/

numbers of lymphocytes and plasma cells are present in the intestinal mucosa of conventionally reared animals, and this makes it difficult to detect significant changes in their numbers or morphology in experimental situations. I tried to overcome this problem by preparing sterile "antigen-free" intestine-grafts of foetal intestine, heterotopically implanted in adult mice of the same strain. These grafts took, grew, resembled normal small intestine in many respects, and so were suitable for use in a variety of immunological experiments.

Design of the experiments

The influence of the presence of antigen within the gut lumen, and the effect of age on the IE lymphocytes, were investigated by comparing antigen-free grafts with normal intestine of the same age-exposed to intraluminal antigens of foods, parasites and microorganisms. Also I examined a small group of histological preparations of germ-free mouse intestine exposed only to intraluminal antigens of foods and killed microorganisms.

Thymus-dependent and thymus-independent lymphocytes were detected by comparing intestine of immunologically intact mice with intestine of mice which had been "thymus-deprived" by neonatal thymectomy or by adult thymectomy and irradiation. Also the traffic to the gut of T, B and normal (T+B) lymphocytes was studied by autoradiographic tracing of intravenously injected labelled lymphocytes.

Rejection of allografts of small intestine

The technique of heterotopic implantation of foetal intestine was successful even when graft and host were of different inbred strains. Allografts of foetal mouse intestine became vascularised and grew normally for a few days, but thereafter were rejected. The rejection process would, in any case, prove an interesting subject for morphological evaluation, as an example of how a local cell-mediated immune reaction can damage the intestine. In addition, this work now has clinical relevance for to date there have been seven reported cases of attempted small-intestinal allotransplantation in man (Fortner et al, 1972) yet the morphological features and time course of rejection of small intestine have still to be adequately defined.

Lymphoid cells of the mammalian small intestine are located in three different sites. There are organised subepithelial aggregates of lymphoid tissue (Peyer's patches and solitary lymphoid nodules); single cells scattered throughout the subepithelial connective tissue (lamina propria lymphocytes, plasma cells, etc.); and single lymphoid cells found between the epithelial cells which line the intestinal lumen (intraepithelial or IE lymphocytes, eosinophils). In this chapter the published work on the IE lymphocytes is reviewed, together with a brief description of Peyer's patches and lamina propria cells. This latter is intended to form a foundation for interpretation of some of the experimental results.

Chapter 2

Intraepithelial lymphocytes and the morphological basis of intestinal immunity

Intraepithelial lymphocytes of the small intestine

Eberth was the first author to report the presence of leucocytes within the columnar epithelium of the small intestine (Eberth, 1864); shortly thereafter this finding was confirmed (von Hessling, 1866; Eimer, 1867; von Arnstein, 1867; Paneth, 1888). These and subsequent workers agree that the intraepithelial cells are usually lymphocytes (reviewed by Wolf-Heidegger, 1939; Andrew and Sosa, 1947) although eosinophils are sometimes present (Andrew and Sosa, 1947; Palay and Karlin, 1959; Back, 1972a).

Morphology of IE lymphocytes. These cells have very irregular outlines, scanty pale cytoplasm and densely staining nuclei. They often appear amoeboid and the term "Zellenwanderung" (Stenqvist, 1934) aptly describes their appearance, wandering within the epithelium. Their fine structure has been described in two publications, with surprisingly variant findings (Andrew, 1965; Meader and Landers, 1967). Both papers agree that most lamina propria lymphocytes have dense nuclei and granular cytoplasm with some rough endoplasmic reticulum, ribosomes, lysosomes, mitochondriae and a well developed Golgi apparatus. Meader and Landers considered that, in passage across the basal lamina, the general configurations of the cells were altered but they appeared/

appeared otherwise normal. Within the epithelium the cells were distorted by surrounding structures and the numbers of lysosome-like bodies were increased, but in other respects they were similar to lamina propria lymphocytes. Andrews described in detail a process of so-called "transformation" of lymphocytes within the epithelium. Lymphocytes were often enlarged with few mitochondriae. He observed pyknosis and fragmentation of nuclei and sometimes noticed whole lymphocytes which were being extruded into the lumen. These changes were most obvious in the crypts and his electron microscopic findings confirmed his previously reported light microscopic observations (Andrew and Andrew, 1945; Andrew and Collings, 1946; Andrew and Sosa, 1947).

Phylogeny of IE lymphocytes. These have been identified in many species including birds, fish, reptiles and amphibia (von Arnstein, 1867; Beguin, 1904; Fichtelius et al, 1969a; Back, 1970a, 1970b, 1972a, 1972b), and many mammals - mouse, rat, rabbit, guinea pig, cat, dog and man (Eberth, 1864; von Arnstein, 1867; Wolf-Heidegger, 1939).

Quantitation of IE lymphocytes. Several authors have attempted to quantitate the IE lymphocytes, usually by counting numbers of epithelial cells and lymphocytes in the same microscopic fields, /

fields, and expressing the results as number of lymphocytes per 100 or 1000 epithelial cells. These values are summarised in Table 2.1.

Relationships between IE lymphocytes and epithelial cells. In many species the IE lymphocytes are concentrated in the basal part of the epithelium, ie between the basal lamina and the level of epithelial cell nuclei. This has been noted in the mouse, rat, hamster and man (Paneth, 1888; Kelsall, 1946; Darlington and Rogers, 1966; Meader and Landers, 1967). The latter three groups of workers formally counted the supranuclear, nuclear and infranuclear lymphocytes and agree that between 95% and 98% of IE lymphocytes are in the basal part of the epithelium. However, in the frog and cat, lymphocytes are found at all levels of the epithelium (von Arnstein, 1867; Wolf-Heidegger, 1939).

For a century there has been dispute as to whether the lymphocytes are all extracellular (ie between epithelial cells) or are lying in vacuoles within epithelial cells. Some workers were firmly convinced that the lymphocytes were extracellular, and lay in deep niches in adjacent epithelial cells (von Schaffer, 1936; Wolf-Heidegger, 1939; Meader and Landers, 1967). Others considered that lymphocytes could be found/

found both between and within epithelial cells (Eberth, 1864; von Arnstein, 1867; Shields et al, 1969) and some were equally convinced that the majority of lymphocytes were intracellular (Andrew and Andrew, 1945; Andrew, 1965). Even electron microscopy has not resolved this question.

Mitosis and DNA synthesis of IE lymphocytes. Andrew and Andrew reported that there were many lymphocytes in mitosis within the epithelium of mouse small intestine, especially in the crypts (Andrew and Andrew, 1945). Once again, this is at variance with the work of authors who found no IE lymphocytes in mitosis (Kelsall and Crabb, 1959), or others who reported only 3 mitotic figures in 1,600 lymphocytes examined (Darlington and Rogers, 1966). It is likely that Andrew and Andrew mistook tangentially cut epithelial cells for lymphocytes, for electron microscopic autoradiography has shown that most dividing crypt cells are undifferentiated epithelial cells, with only an occasional lymphocyte found in DNA synthesis (Kataoka, 1970). Now it is generally agreed that a small proportion of IE lymphocytes is in DNA synthesis as measured by the incorporation of ^{3}H-thymidine (Darlington and Rogers, 1966; Fichtelius, 1968; Lemmel and Fichtelius, 1971; Back, 1972b). The pattern of labelling of lymphocytes appears to be completely independent/

independent of epithelial cell labelling. Darlington and Rogers found that mouse IE lymphocytes divided at a much slower rate than columnar epithelial cells, and that the dividing lymphocytes showed no preference for any particular position on the villus. They calculated that the IE lymphocytes, or their parent population, had a mean generation time of around 17 days (Darlington and Rogers, 1966).

Influence of age on the numbers of IE lymphocytes. This has been investigated to a limited extent in mice and chickens (Andrew and Sosa, 1947; Fichtelius et al, 1968b; Back, 1972a). The results, summarised in Table 2.2, show that in both these species there are few IE lymphocytes in the neonatal period.

Influence of antigenic stimulation on the numbers of IE lymphocytes. Several workers have mentioned the presence of IE lymphocytes in intestines which have been protected from or deprived of antigenic stimulation. IE lymphocytes are present in 100 mm human foetuses (Fichtelius, 1969a, in discussion), in germ-free rats (Fichtelius, 1968) and in germ-free piglets (Good, 1969). This latter observation confirms an early report that IE lymphocytes were present in/

in "almost germ-free" guinea pigs (Stenqvist, 1934). Thus it is agreed that IE lymphocytes are present in the germ-free state but there is dispute as to whether the absence of live gut microorganisms influences their numbers (Fichtelius, 1968).

Thymus-dependence of the IE lymphocytes. No-one has, as yet, directly traced thymus or bone-marrow derived cells directly to the intraepithelial site, although there is a single report that ^{3}H-thymidine labelled lymphocytes from rat lymph home to the small bowel epithelium as well as to the lamina propria. (Goldschneider and McGregor, 1968a). Nevertheless there is circumstantial evidence that some IE lymphocytes are thymus-dependent, for the numbers of IE lymphocytes are reduced in neonatally thymectomised mice (Fichtelius et al, 1968b) and in thymectomised, irradiated chickens (Back, 1970a). Back also reported that IE lymphocyte counts were increased after bursectomy and irradiation and considered this as evidence that some IE lymphocytes could act as a bursa-equivalent, even in chickens (Back, 1970b).

Origins and fate of IE lymphocytes. There have been several unusual theories as to the derivations, fate and functions of the IE lymphocytes. It has been suggested that epithelial cell nuclei divide, and lymphocytes are produced from these bi-

binucleate cells (Grumhagen, 1887); that lymphocytes migrate into the epithelium and rejuvenate the ageing enterocyte nuclei by a process of "caryo-anabiosis" (Guieysse-Pellissier, 1912); and that mesenchymal cells wander into the epithelium, enter the cytoplasm and serve as the source of enterocyte nuclei (Goldner, 1929). The trephocyte theories discussed below probably developed from these lines of thought. In general, though, most authors agree that lymphocytes cross the basal lamina, so probably enter the epithelium from the lamina propria. Indeed, Meader and Landers claimed that they could identify lymphocytes both entering and leaving the epithelium (Meader and Landers, 1967). They based this inference on two ultrastructural features - the direction in which the basal lamina and adjacent collagen fibres were ruptured, and the situation of the lymphocyte nucleus which is reputed to lie at the front of a motile lymphocyte (Lewis, 1931).

Once within the epithelium there are several possible fates of the lymphocytes. It has been suggested that the cells degenerate within the epithelium, probably as a result of performing some defensive, antibacterial function (de Winiwarter, 1930; Hellman, 1934; Andrew and Andrew, 1945). Several workers have found lymphocytes lying free in the intestinal fluid (Bunting and Huston, 1921; Jassinowsky, 1925; Stenqvist, 1934) and/

and deduced either that lymphocytes performed their defensive function within the gut lumen (Stenqvist, 1934) or that the gut was the "lymphocyte graveyard" (Zuntz, 1905; Bunting and Huston, 1921). However this latter theory was refuted by Erf who showed that lymphocytes could disappear from the blood even when the entire small and large intestines had been resected (Erf, 1940). Now it is known that lymphocytes leave the blood to recirculate from blood to lymph in the lymphoid tissues (Gowans and Knight, 1964). Another possibility is that the cells re-enter the lamina propria. Their persistence in the basal part of the epithelial cell layer has been taken as support for this theory (Paneth, 1888; Kingsbury, 1944; Kelsall, 1946) and Kelsall further suggested that they transform into lamina propria plasma cells.

All of these theories have been based on purely morphological grounds, so the fate of the IE lymphocytes must still be considered an open question.

Recent theories of the function of IE lymphocytes. The idea that the gut is the site of excretion of lymphocytes, and the possibility that IE lymphocytes have a defensive role, have been mentioned above. In the 1950s, there was a surge of interest in the idea that lymphocytes might act as trephocytes - ie transfer nutrients to other cells by means of dialysis, cytoplasmic budding/

budding or perhaps by cytolysis. Kelsall and Crabb pointed out the many features of IE lymphocytes which suited them to the role of trephocytes: high content of nucleic acids, RNA and histones; considerable motility and capacity to enter the epithelial cell substance; rarity of mitosis in vivo, ie the rarity of an energy-utilising process, so that their energy could be stored and transported to recipient cells (Kelsall and Crabb, 1959). Loutit was also impressed by the trephocyte potential of lymphocytes and pointed out the syndrome of runt disease (which occurs in several types of lymphocyte depletion) as an obvious result of the lack of lymphocyte trephocytes (Loutit, 1962, 1963). Although this theory aroused considerable interest ten years ago, recent advances in knowledge of the nature and functions of lymphocytes have rendered it obsolete.

In 1967, Fichtelius originated yet another theory of the functions of IE lymphocytes, which he called "theliolymphocytes". He had undertaken an extensive phylogenetic study of lympho-epithelial organs (reported by Fichtelius et al, 1968a) and found lymphocytes in the gut epithelium of all species studied. Also he considered that the reported rates of DNA synthesis in these cells was exceptionally high, similar to the high values for the central lymphoid organs, the thymus and bursa of Fabricius. He considered that these facts were consistent with the theory that the theliolymphocytes are still evolving as a "bursa-equivalent"/

"bursa-equivalent" in mammals and perhaps in other vertebrates (Fichtelius, 1967). In a series of papers he reported that some 3% of rat theliolymphocytes were in DNA synthesis, with no difference between germ-free and conventional rats (Fichtelius, 1968); their numbers are reduced in neonatally thymectomised mice (Fichtelius et al, 1968b); theliolymphocytes are radio-sensitive but their parent population is radioresistant (Fichtelius, 1969b); their numbers are slightly increased in hibernating squirrels (Fichtelius and Jaroslow, 1969); and that most jejunal theliolymphocytes are short lived (Lemmel and Fichtelius, 1971). Apart from the rat experiment, which was based on only 8 animals, none of these papers relate directly to his theory. Also the strength of his argument for theliolymphocytes as a bursa equivalent was reduced by his simultaneous publication of work which suggested that Peyer's patches are a bursa equivalent (Fichtelius et al, 1968a; Fichtelius, 1969a). Finally he extended his theory to include skin (Fichtelius, 1971) and eventually a complete spectrum of lymphoepithelial organs including salivary glands, bronchi, mammary glands and rectal tonsil (Fichtelius, 1969c; Fichtelius et al, 1969b; Fichtelius 1970) - based entirely on morphology.

Nevertheless, despite the lack of evidence and over-enthusiastic propagation, /

propagation, the basis of Fichtelius's theory is still interesting. He points out that primitive antigen exposure was at the body surfaces, skin and gut. At first, epithelial cells were directly antigen-reactive; perhaps they have continued to instruct the lymphocytes and antibody-forming cells which evolved later. This is certainly true of the thymus and bursa of Fabricius, which are derived from epithelial surfaces; their presence still does not preclude a continuing direct instructive function of the epithelia.

Peyer's patches

These were described by Peyer in 1677. They have been found in all mammals studied, but not in other vertebrates (Fichtelius et al, 1968a). Their number varies considerably from around 12 in the mouse (Sobhon, 1971) and 20 in the rat (Hummel, 1935) to 200 in man (Cornes, 1965).

Structure of Peyer's patches in mice. This description is based on personal observations and on two recent reports of Peyer's patch morphology (Sobhon, 1971; Chin and Hudson, 1971). Peyer's patches extend through lamina propria and submucosa of the small intestine. The overlying epithelium is flattened and heavily infiltrated by lymphocytes. Crypts and villi are sparse/

sparse, and the patches are in direct contact with the muscle layer. The structure of a typical patch is illustrated diagrammatically in Fig. 2.1. There are four distinct areas - nodules (N), some of which contain germinal centres (GC), separated by interfollicular areas (IF) and with discrete subepithelial zones (SE) between the nodules and overlying epithelium. Typical post-capillary venules are present in the interfollicular areas and they resemble their counterparts in the thymus-dependent areas of lymph nodes (Parrott et al, 1966; Ropke et al, 1972). There are also lymphatic vessels located under the epithelium and adjacent to the external muscle layer. The majority of Peyer's patch lymphoid cells are lymphocytes, but the subepithelial zone contains many macrophages and plasma cells. Also, lymphocytes can be seen in transit across the basal lamina between the subepithelial zone and the epithelium.

Functions of Peyer's patches. Although several groups have suggested that Peyer's patches are the bursa equivalent of bursaless vertebrates (Cooper et al, 1966; Perey et al, 1968; Fichtelius et al, 1968a), there is increasing evidence that they behave like peripheral lymphoid tissue. Peyer's patches of young mice are mainly comprised of thymus-derived lymphocytes (Joel et al, 1971, 1972; Chanana et al, 1973). In older mice they contain/

contain both thymus derived and bone-marrow derived lymphocytes (Evans et al, 1967) and depleted thymus-dependent areas can be clearly identified in the Peyer's patches of athymic mice (de Sousa et al, 1969). Although Peyer's patches are small in germ-free animals, immunisation or infection of these animals results in enlargement of Peyer's patches, with germinal centre formation, suggesting that their usual large size is related to antigenic stimulation (Cooper et al, 1968; Pollard and Sharon, 1970). Furthermore, Peyer's patches have been shown to resemble spleen rather than thymus in the immune response, as assessed by the numbers of antigen-reactive cells and antibody-producing cells after antigenic stimulation (Armstrong et al, 1969; Henry et al, 1970). It seems very likely, although not yet proven, that in normal mammals, Peyer's patches participate in immune reactions, both local and systemic.

Lymphoid cells of the lamina propria

Textbook descriptions of the lamina propria tend to underestimate the amount and importance of the lymphoid cells. Bloom and Fawcett define the lamina propria as "tissue which fills the spaces between the glands of Lieberkuhn and forms the core of the villi". Ham's description is of "loose ordinary connective tissue with lymphatic tendencies". In fact, most lamina propria cells are of the lymphoid series.

Mononuclear phagocytes (macrophages) form a network in the lamina propria. Like other cells of the reticuloendothelial system, they have been shown to ingest intravenously administered particles (flagellae) and protein (peroxidase) (Dobbins et al, 1968; Hunter, 1972). Macromolecules are transported from the gut lumen to the lamina propria via intracellular enterocyte vacuoles (Cornell et al, 1971; Walker et al, 1972) and it seems likely that these macromolecules are phagocytosed and processed by the lamina propria macrophages.

Lamina propria lymphocytes morphologically resemble those of the peripheral blood (Andrew, 1965; Meader and Landers, 1967). Many are in DNA synthesis (Olsen, 1969), and this may be related to the observation that large lymphocytes of rat lymph (which are in DNA synthesis) home preferentially to the lamina propria of the gut, and can there be identified as lymphocytes or plasma cells. (Gowans and Knight, 1964; Goldschneider and McGregor, 1968a, 1968b; Delorme et al, 1969; Hall and Smith, 1970; Howard et al, 1972).

Most plasma cells of the lamina propria contain Immunoglobulin A, and specific antibodies in intestinal secretions are usually of IgA class (Tomasi and Bienenstock, 1968). Their numbers are reduced in germ-free mice (Crabbe et al, 1968) but oral administration of antigen (in germ-free mice) results in the appearance/

appearance of lamina propria plasma cells which contain specific antibody, of IgA class (Crabbe et al, 1969). They are probably derived from immunoblasts of lymph (see above) and it is likely that they synthesise and secrete antibody to various antigens normally present in the gut lumen.

Mast cells and eosinophils are also present in the lamina propria. These cells are occasionally seen crossing the basal lamina and can be found in an intraepithelial site (Toner, 1968).

Table 2.1 Quantitation of IE lymphocytes in the small intestine.

Author	Species	Area counted	IE lymphocytes per 100 epithelial cells
Fichtelius et al (1969a)	various fish	crypts & villi	0.3 - 49
	amphibia	" "	5 - 14
	reptiles	" "	1 - 25
	birds	" "	1 - 12
Back (1972a)	chicken	tops of villi	10.0
Andrew (1965)	mouse	crypts	6.5 - 16.7
		villi	4.5 - 36.5
Darlington and Rogers (1966)	mouse	villi	9.3
Fichtelius (1968)	rat	villi-jejunum	9.4
		villi-ileum	10.5
Nakano (1933)	guinea pig	villi	12.1 - 12.9
Bunting and Huston (1921)	rabbit	villi	40 or 50 at top of each villus
Shields et al (1969)	man	crypts & villi	6.4 - 8.7 (but excluded "intracellular" lymphocytes)

Table 2.2 Small intestinal IE lymphocyte counts related to age.

Author	Species	Age	IE lymphocytes per 100 epithelial cells
Andrew and Sosa (1947)	mouse	4 - 17 days	some in crypts
		17 - 20 days	a few in villi
		30 days	many in villi
Fichtelius et al (1968b)	mouse	2 weeks	0.5
		3 weeks	4.9
		2 months	6.4
		5 months	4.5
Back (1972a)	chicken	neonatal	1.0
		1 week	3.0
		7 weeks	6.3
		9 weeks	14.7
		18 months	10.0

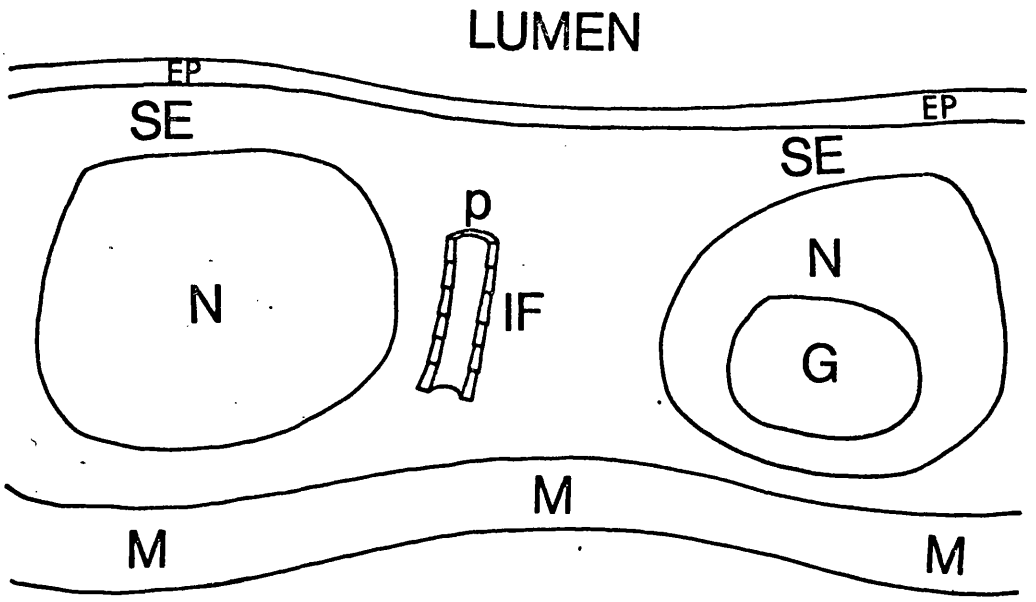

Fig. 2.1 The structure of a typical Peyer's patch in an adult mouse.

EP	-	epithelium
SE	-	subepithelial lymphoid tissue
N	-	nodular lymphoid tissue
G	-	germinal centre
M	-	muscle
IF	-	interfollicular zone
P	-	post-capillary venule

Chapter 3

Materials and Methods

Animals

Mice used for the experiments were of the inbred strains CBA, BALB-c and C3H-Bi, maintained in the University Department of Bacteriology and Immunology, Western Infirmary, Glasgow.

Rats were of the inbred strains Wistar and SA, and outbred Charles River rats, all maintained in this department.

Diet. All animals had free access to tap water and to the rodent diet FFG(M).

Newborn mice were kept in the mother's cage for three weeks after birth. They were suckled by the mother for these three weeks, but from the age of 16 days or so, they nibbled pieces of food pellet.

Anaesthesia

Most operations, and irradiation, were carried out on mice which had been sedated with Hypnorm (Crown Chemical Company Ltd.). Supplementary ether anaesthesia was used as required, and ether alone was used for brief procedures such as intravenous injections.

Hypnorm was diluted in distilled water - 1:9 Hypnorm:water. Groups of about 10 mice were sedated with an intraperitoneal injection of diluted Hypnorm, 0.1 ml per 10 g. body weight. For rats, the optimal intraperitoneal dose was found to be .07 ml per 10 g. After 20 minutes, the animals lie quietly and can be shaved, taped to the irradiation tray, etc. They have a slow respiratory rate, which is an advantage for thymectomy; they still react to painful stimuli. This tranquil state lasts for about 2 hours.

Killing of animals

Young mice were killed by cervical dislocation.
Older mice, and rats, were killed by ether overdosage.
Pregnant mice (for donors of foetal gut) were anaesthetised with ether, the foetuses removed from the uterus, and the mouse then killed by ether overdosage.

Preparation of thymus-deprived mice

CBA mice were thymectomised at age 6-8 weeks; 2-4 weeks later they were given 850r total body irradiation, and reconstituted with bone marrow cells in one of two ways. In most animals,

5-10/

5-10 x 10^6 CBA bone marrow cells were injected intravenously. For the cell traffic and some of the allograft rejection experiments, bone marrow of the lower part of the hind limbs was protected by lead shielding, thus obviating the need for isogeneic bone marrow reconstitution.

Thymectomy. Mice were anaesthetised with Hypnorm, supplemented by ether. The anterior chest wall was shaved and the animal placed supine on a cork board. The four limbs were stretched out and immobilised with adhesive tape. The chest wall was sterilised with a few drops of 70% ethyl alcohol. A longitudinal incision was made in the skin over the anterior half of the sternum. The sternum was cut transversely at the junction of the anterior and middle thirds, exposing the thymus in the upper mediastinum. Both lobes of the thymus were sucked out using a flat-tipped bent glass cannula attached via a T-piece to a vacuum pump. The incision was closed with 2 metal clips.

Irradiation. Mice were sedated with Hypnorm, then taped to a steel tray with micropore tape (up to 24 per tray, 20 x 20 cm). 850r whole body irradiation was given by using either a ^{60}Co source (Orbitron) or by using X-rays at 300 kilovolts, and 20 milliamps, with a Thoraeus filter (Resomax).

Irradiation was performed by Miss J. Laurie.

Bone-marrow shielding. When the mice were sedated, the two hind limbs were taped together with micropore tape. A strip of soft lead, 2 mm thick, 1 x 4 cm size, was wrapped around the lower parts of the hind limbs, care being taken that the area of the popliteal lymph nodes was not shielded.

Bone marrow reconstitution. Normal CBA mice, aged 2-3 months, were killed, femora and humeri removed, cleaned, and both ends of each bone cut off with a scalpel. Marrow was washed from the central cavity by the injection of 0.1 ml tissue culture medium (Eagles MEM) through the marrow cavity, via a 20 or 21 gauge disposable needle. The cells were further dispersed by drawing them into and out of a disposable syringe, through a 23 gauge needle. They were washed 3 times in Eagles medium (centrifuged for 5 minutes at 1200 G, supernatant discarded, cells resuspended in 10 ml medium). The washed cells were counted in a Nebauer chamber, and between 5 and 10×10^6 cells, in a volume of 0.1 ml, were injected intravenously, into the femoral vein, with a 30 gauge steel needle.

Preparation of foetal intestine for grafting

A mouse, judged to be about 18 days pregnant, was anaesthetised with ether; the abdomen was opened and the foetuses were removed from the uterus (the exact gestational age could be determined/

determined by histological examination of one of the foetuses). Each foetus was pinned out on a cork board, the intestinal tract was removed from the abdomen and placed in ice-cold phosphate-buffered saline (PBS) at pH 7.2. The coils of intestine were teased apart and the fragile mesentery removed. Stomach, small intestine, caecum and colon could be easily identified morphologically. The material to be grafted (usually small intestine) was selected, transferred to another dish of cold PBS and cut into fragments about 10 mm in length.

Implantation of grafts of intestine under the kidney capsule

Recipients aged $2\frac{1}{2}$ to 6 months were anaesthetised with Hypnorm and ether. The skin was shaved, the mouse placed on its side on a cork board and immobilised with strips of adhesive tape. A vertical incision was made in the lumbar region and the underlying kidney gently pulled out of the abdomen and held in place by grasping the lower part of the renal mesentery with the tips of fine Spencer-Wells forceps.

A longitudinal incision was made in the renal capsule with a rounded scalpel blade. A soft polyethethylene cannula attached to a syringe (eg a Braunula intravenous cannula size 0) was used to lift a piece of donor intestine from the PBS . Surface tension held the graft on the tip of the cannula (Fig. 3.1). The edge of the incised capsule was lifted up with fine forceps and the foetal intestine/

intestine placed under the capsule (Fig. 3.2). The soft tip of the cannula was used to push the graft well away from the incision. If there was any bleeding from the capsular incision, a small piece of Surgicel was placed over the bleeding point. The kidney was replaced within the peritoneal cavity and the abdominal muscle layer closed with one or two fine silk sutures. The skin incision was closed with a metal clip.

Implantation of grafts of intestine in millipore chambers within the peritoneal cavity

Cylindrical millipore chambers were constructed from two millipore discs (pore size 0.22μ) and a perspex ring, sealed with millipore glue. A fragment of foetal intestine, with 0.1 ml PBS, was put inside each chamber. Recipient mice were anaesthetised and immobilised, supine, with strips of adhesive tape. The skin of the abdomen was shaved and a longitudinal incision made in the midline (2 cm long). The millipore chamber was slipped into the peritoneal cavity; the muscle layer closed with black silk sutures, and the skin incision closed with metal clips.

Tracing of carbon

In order to identify the lymphatic drainage of the renal subcapsular site, in 4 mice, with established grafts, a suspension of/

of carbon particles was injected into this site. The right or left kidney was exposed by a loin incision, the kidney was immobilised with Spencer-Wells forceps and 0.02 ml of a carbon suspension was injected into the angle formed by the graft and the capsule. Care was taken not to spill carbon onto the surface of the kidney; a fine needle (26 gauge) was used, and after the needle had been withdrawn the puncture site was sealed with a drop of Whatman's tissue glue. 24 hours later the mice were killed and the distribution of carbon was checked by careful autopsy.

Bacteriological culture of grafts

Four grafts, which had been in situ for 5 months, were cultured in order to ensure that they were, indeed, sterile. Mice were killed, pinned on a cork board, and in each mouse the skin was drenched with 70% ethyl alcohol and cut open. The peritoneum was lifted and incised with sterile instruments; the kidney with graft was removed with another set of sterile instruments, and the graft was cut away from the kidney with a sterile scalpel. The grafts were homogenised and cultured for 3 days (by Dr. H. Singh) aerobically and anaerobically on blood agar, on MacConkey's medium and in Robertson's meat medium.

Nippostrongylus brasiliensis infection of mice

Nippostrongylus larvae were supplied by Dr. E. E. E. Jarrett 1000 larvae were injected subcutaneously into each mouse. Larvae migrate via lymphatics, blood, lungs, bronchi, oesophagus and stomach to reach the small intestine about 3 days after injection. Mice were killed 6 days after injection, and the mesenteric lymph node cells were used for cell traffic experiments.

Induction of oxazolone sensitivity

0.02 ml of a 3g/100 ml solution of oxazolone in absolute ethyl alcohol was painted on each ear. Mice were killed 3 days later and auricular lymph node cells were used for cell traffic experiments.

In vivo labelling of dividing epithelial cells

Proliferation kinetics of cells can be studied by ^{3}H thymidine labelling of dividing cells and subsequent autoradiography (Leblond and Messnier, 1958). 4 mice, with grafts of small intestine, were given an intraperitoneal injection of 100 µc ^{3}H thymidine (Radiochemical Centre, Amersham). 2 mice were killed 2 hours later, and 2 at 24 hours. Autoradiography

of/

of intestine, and grafts of intestine, was performed as described below.

In vivo labelling of lymphocytes

Young mice (10-15 g.) were injected twice daily for 3 days, with 10 μc of ^{3}H thymidine. Cell suspensions of thymus and mesenteric lymph nodes were washed and used as donor cells for cell traffic experiments.

In vitro labelling of lymphocytes

Mice were killed by ether overdosage, organs (thymus, mesenteric or auricular lymph nodes) from several mice were pooled, and suspensions of cells were prepared as follows. The organs were teased apart in about 1 ml of Eagle's medium, and further dispersed by aspiration through 20, 21 then 23 gauge needles. Cell suspensions were incubated in a shaking water bath for $1\frac{1}{2}$ hours, in 5 ml of PBS with ^{3}H thymidine at a concentration of 2.5 μc per ml, or ^{3}H adenosine at a concentration of 10 μc per ml. After incubation, the cells were washed three times with Eagle's medium, suspended in a volume of around 1 ml of PBS and counted, in a Nebauer chamber, before injection into the femoral veins

of/

of recipient mice (as described above for injection of bone marrow cells).

Removal of tissues for histology

Organs for histological examination were removed within 2 or 3 minutes of the animal's death. When appropriate, spleen, thymus, mesenteric, cervical, axillary and/or inguinal lymph nodes were dissected out and trimmed free of fat, mesentery, etc. In young mice (up to 4 weeks of age) the small intestine was dissected free of mesentery and wound around a small cone of paper before fixation, so that after histological processing a slide contained 10 or more sections of intestine, at different levels. In older mice, 1 cm segments of proximal jejunum, distal jejunum and distal ileum were taken for histological examination. Grafts of intestine, previously implanted under the kidney capsule, could be identified at postmortem examination as rounded white swellings on the surface of the kidney. These grafts were dissected out, with a small amount of underlying renal tissue.

Routine histological processing of mouse and rat tissues

All tissues were fixed in formol saline, embedded in wax, and several sections, 5μ thick, were stained with haematoxylin and/

and eosin (H & E) and methylgreen pyronin (MGP). Most of the grafts of intestine were sectioned at 3 levels, ie 3 groups of sections were cut, with 100μ distance between each group. Mr. H. Cairns carried out all histological processing.

Histological processing of human jejunal biopsies

Specimens of upper jejunal mucosa were obtained with a suction-biopsy capsule.

The capsule was immediately withdrawn, the specimen of jejunal mucosa removed, placed on a small piece of filter paper (villi upwards) and fixed in 20% formalin. After fixation the mucosal surface was examined with the dissecting microscope, then the tissue was embedded, 7μ sections were cut at 3 levels (aligned in a plane vertical to the mucosa) and sections were stained with H & E.

Dissecting microscopy of animal tissues

Small intestine, and grafts of intestine, were dissected out, cut longitudinally and gut or graft contents were removed by shaking the tissue in a beaker of saline. The material was fixed in formol saline to which had been added a few drops of crystal violet. The villous pattern was examined with a dissecting microscope, using incident light.

Electron microscopy

The electron micrographs were prepared by Dr. P. G. Toner. Specimens of human jejunum, mouse small intestine and grafts of intestine were cut into fragments 1-2 mm diameter and placed in fixative within 30 seconds of biopsy or removal from the animal. After preliminary fixation in 2% phosphate-buffered glutaraldehyde the specimens were post fixed in osmium tetroxide and embedded in Araldite. Sections were cut on the LKB Ultrotome, stained with uranyl acetate and lead citrate and examined in the Siemens Elmiskop 1A electron microscope.

Autoradiography

This was carried out by Mrs. C. P. Pearson, using the dipping method (Kopriwa and Leblond, 1962). 10 ml of Ilford KS emulsion was melted and diluted with 30 ml distilled water, at 45°C. Sections (mounted on slides) were dipped into the emulsion, drained, placed in a rack and allowed to dry horizontally for 45-60 minutes. When dry, the slides were transferred to cardboard slide trays, stored, with a dessicant ($CaCl_2$) in a lightproof box at 4°C for several weeks. Slides were developed in Kodak D-19B for 5 minutes, rinsed in tap water and fixed in Amfix for 4 minutes. They were then rinsed for 30 minutes in slow/

slow-running tap water, and stained through the emulsion with MGP.

Microscopy

All the histological preparations were examined with a Watson Microsystem 70 microscope, using x4, x10 and x40 objectives. In addition, lymphocytes were quantitated as described below.

Quantitation of IE lymphocytes of intestinal villi

Differential counts of nuclei were carried out in the epithelium covering the villi. Counts were made in areas of the slide where the tissue was well orientated, the plane of the section passed vertically through the epithelium and the basement membrane was easily identified. The epithelium was examined using the x40 objective and the nuclei of epithelial cells, goblet cells, and lymphocytes could be readily distinguished and counted (Fig. 3.3). When the section contained only part of a nucleus, this was included in the count if it could be firmly classified as epithelial or lymphocyte. Small, unclassifiable nuclear fragments were ignored. Lymphocyte nuclei were characteristically basally situated and they had dense nuclear staining. The nuclear shape was often very irregular and the surrounding lymphocyte cytoplasm was very pale. A single field usually contained 30-40 epithelial/

epithelial cells and a variable number of IE lymphocytes. Nuclei of goblet cells were not counted; IE eosinophils and lymphocytes in transit across the basal lamina were noted, but not included in the final calculation of the lymphocyte count. For each specimen, at least 500 cell nuclei (epithelial and lymphocyte) were counted. The result was expressed as the number of IE lymphocytes per 100 villous epithelial cells. No difficulty was encountered in carrying out the differential counts as long as care was taken to count areas of the slide where the plane of section was vertical to the epithelium.

Counts of ^{3}H labelled cells in lymphoid organs and intestine

Most of these counts were carried out by Professor D.M.V. Parrott, with a Leitz Laberlux microscope, under oil, using a x95 objective. A grid eyepiece was used, and, for each tissue, the number of labelled cells per 10 grid squares(of the eyepiece) were counted; cells with more than 10 silver grains in the overlying emulsion were considered to be labelled. Labelled cells in villi were found by scanning about 40 villi under oil; the rest of the tissue was examined under a flatfield x40 objective.

Gifts of histological material

Slides of intestine from three groups of animals were kindly gifted/

gifted or loaned to me.

(1) From Dr. D.M.V. Parrott: H & E stained sections of small intestine from neonatally thymectomised C3H and F1 C3HXC57B1 mice, and their littermate controls (conventionally reared).

(2) From Dr. J.C. Salomon: H & E stained sections of intestine from germ-free C3H mice, some of which had been neonatally thymectomised.

(3) From Dr. M. de Sousa: H & E stained section of a Peyer's patch from a congenitally athymic mouse.

Immunoglobulin assay

Immunoglobulins were assayed by radial immunodiffusion in the serum and tissue homogenates of a group of 9 mice. Antisera to mouse IgG_1, IgG_2, IgA and IgM were purchased from Flow Laboratories. For each assay plate 3 ml of 1% agarose in barbitone buffer (pH 8.2) was melted, cooled to 56°C and 0.1 ml antiserum added. The agar was then poured into a flat-bottomed plastic dish and allowed to solidify. 12 holes (3 mm diameter) were punched in each plate, and a 5 µl volume of standard or test solution placed in each hole. Pooled normal mouse serum was used as a standard and taken to have a content of 100 units/ml

of/

of each immunoglobulin. The standard solutions, prepared by dilution of standard serum with PBS, contained from 1 to 100 units/ml. Test sera and homogenates were used neat or diluted 1 in 10 or 1 in 40 for testing.

Mice were bled by cutting the axillary artery and vein in anaesthetised animals. Serum was separated by centrifugation. Tissues were weighed, homogenised in ice cold PBS (100 mg/ml), the suspension centrifuged in a Hawkins microcentrifuge and the supernatant used for Ig assays.

Solutions used

Phosphate buffered saline at pH 7.2.

This was made up by dissolving 8.76 g sodium chloride, 1.42 g disodium hydrogen phosphate and 0.342 g potassium dihydrogen phosphate in 1 litre distilled water.

Barbitone buffer at pH 8.2.

This was made up by dissolving 7.93 g sodium barbitone in about 500 ml distilled water, adding 115 ml 0.1 normal HCl and making up to a final volume of 1 litre with distilled water.

Eagle's minimum essential medium was prepared as described by Paul (1972), and supplied by the Institute of Virology.

Statistics

Means, standard errors, standard deviations, t, and probabilities of differences between groups of values were calculated using a Hewlett Packard calculator. The use of probability paper in the analysis of human jejunal lymphocyte counts is described in chapter 4.

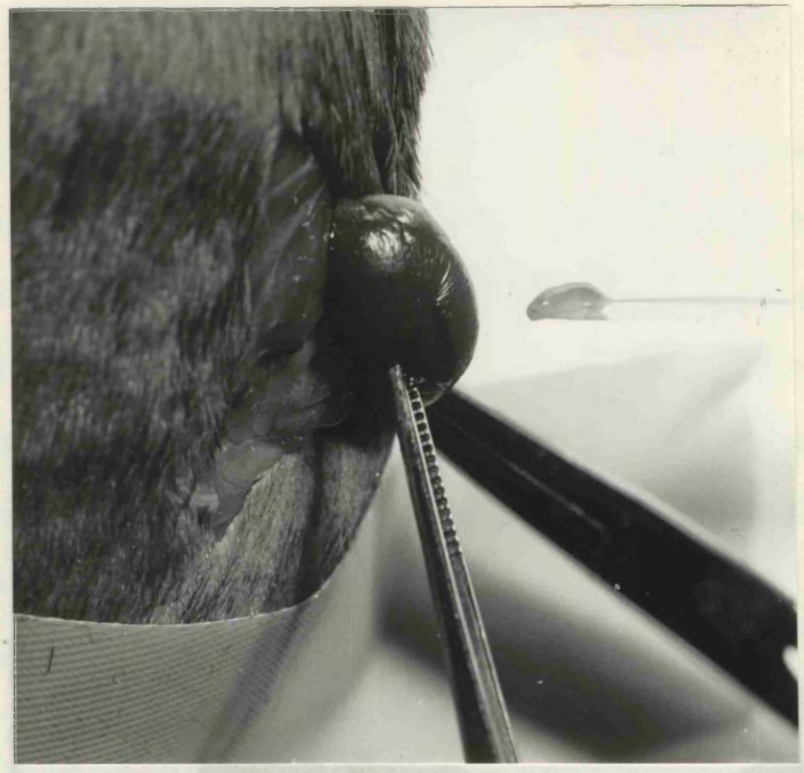

Fig. 3.1 Implantation of a graft under the kidney capsule of an adult mouse. One edge of the incision in the renal capsule is lifted with fine forceps. The material to be grafted is held by surface tension at the tip of a flexible polyethylene cannula. Xc4.

Fig. 3.2 The soft cannula is used to push the graft under the renal capsule, well away from the incision. Xc4.

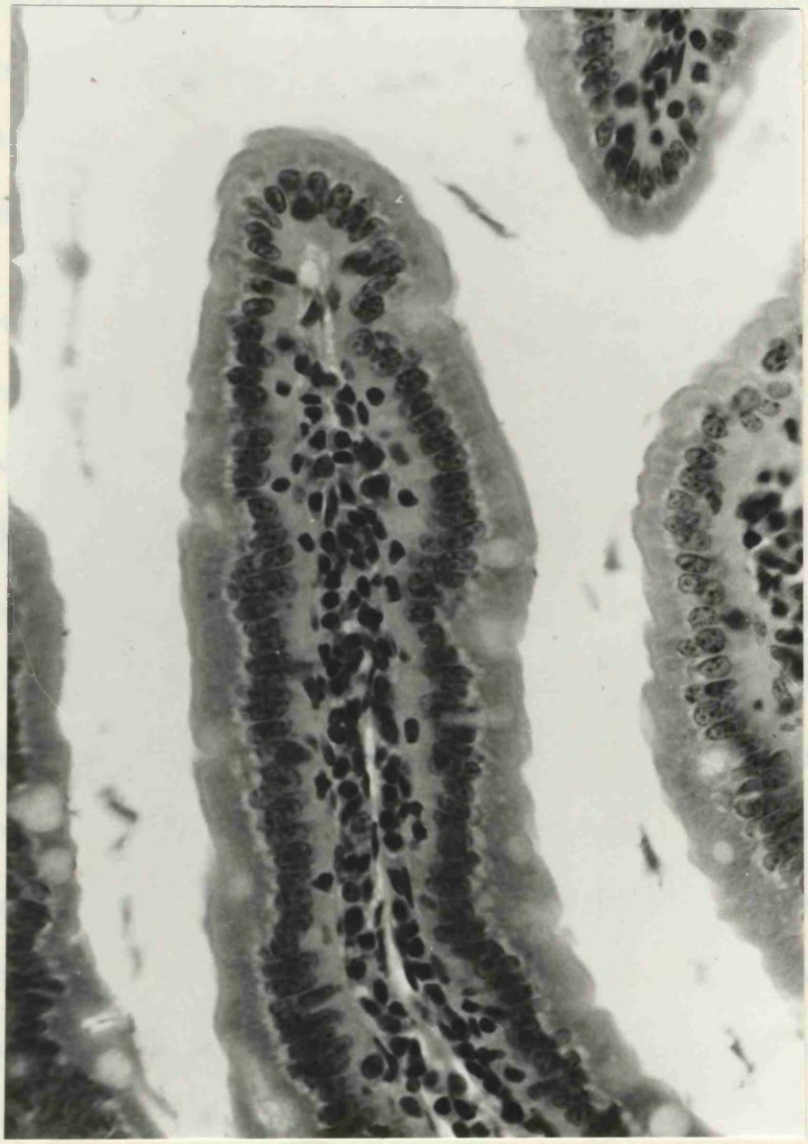

Fig. 3.3 Small intestinal villus of a CBA mouse aged 18 days. Nuclei of epithelial cells and IE lymphocytes can be clearly distinguished.
H & E. x600

Chapter 4

Intraepithelial lymphocytes of human jejunum

Suction biopsy of the small intestine is part of the routine clinical investigation of a patient with diarrhoea, malabsorption or unexplained malnutrition. The abnormality which is detected most frequently is the "villous atrophy" of coeliac disease, in which classically the mucosal surface is convoluted or flat; villi are absent, crypts are long, the epithelium is of irregular cuboidal cells, and the tissue is heavily infiltrated with lymphoid cells (Hourihane, 1966; Cappell and Anderson, 1971).

Between 1962 and 1969, around 1000 jejunal biopsies were performed by the gastroenterologists in the Royal Infirmary, Glasgow (Drs. W.C.Watson, E. Paton, J.D. Maxwell and myself). Drs. D. Murray and A.M.McKay (University Department of Pathology, Royal Infirmary) had classified and stored the histological sections according to the patients' diagnoses and histological abnormalities. I re-examined 200 of these biopsies in order to study the morphology of human IE lymphocytes; to find if it was practicable to quantitate them in routinely processed biopsies; and to determine whether their morphology and numbers were altered in biopsies from patients with ulcerative colitis, Crohn's disease, coeliac disease, autoimmune and other immunological diseases.

In parallel, I carried out a prospective study of the ultrastructure of/

the IE lymphocytes, in collaboration with Dr. P.G. Toner of the University Department of Pathology, Western Infirmary, Glasgow. We planned to describe the ultrastructural features of the human IE lymphocytes, to examine their relationship to adjacent epithelial cells and the basal lamina, and to look for evidence of degeneration of IE lymphocytes.

Patients and specimens studied

Jejunal biopsy specimens were obtained from the first loop of jejunum, with a Crosby or Watson capsule.

For electron microscopy, in 16 cases the diagnosis of adult coeliac disease had been made. In others, the final diagnosis was not of small bowel disease, and biopsies from these patients were of normal appearance. A few specimens of histologically normal jejunum were obtained, at laparotomy, from patients undergoing surgery for duodenal ulcer. In all, 50 specimens were examined.

For light microscopy, 200 biopsies were selected for assessment. 160 were from patients who did not have clinical coeliac disease; dissecting microscopy had shown finger, tongue or leaf shaped villi and villi and crypts had appeared normal histologically. 20 biopsies were from patients with adult coeliac disease taking a normal diet, and 20 from adult coeliac patients taking a gluten-free diet. The diagnosis of coeliac disease was based on two criteria: (1) jejunal histology of subtotal villous atrophy or severe partial villous atrophy and (2) evidence of clinical, biochemical and/or histological improvement when gluten was withdrawn from the diet.

Histology and ultrastructure of IE cells of human jejunum

The various cell types of the intestine were readily identified by their well known morphological and ultrastructural features. They included the absorptive cells and crypt chief cells, the goblet cells, a variety of endocrine cell types and the Paneth cells (Toner, 1968). In addition, there were many intraepithelial cells that did not share the features of the main epithelial cell types. The great majority of these were lymphocytes but occasional IE mast cells and eosinophil leucocytes were seen.

Lymphocytes were usually basally situated and showed considerable variation in cell contours. Some had nearly circular outlines, others were elongated, and others had irregular amoeboid outlines. They had dense nuclei and scanty pale cytoplasm. Ultrastructurally, the nucleus was compact, rather dense, and often indented or slightly irregular in outline. There was often a compact nucleolus (Figs. 4.1 and 4.2). Detailed cytoplasmic features included variable numbers of ribosomes, scanty granular endoplasmic reticulum, a few small mitochondria and a compact Golgi apparatus (Fig. 4.3) with nearby centrioles. Multivesicular bodies were relatively common, as were small dense homogeneous membrane-limited granules which resembled lysosomes/

lysosomes (Figs. 4.1 and 4.2). Occasionally up to 5 or 6 were seen in a single cell. There were occasional thick-walled or coated micropinocytotic vesicles (caveolae) at the lymphocyte surface, similar features being seen at the surface of the columnar cells. Thus, in general the IE lymphocytes resembled their counterparts in other lymphoid tissues and in blood, but, as a group, they were larger than the average blood lymphocyte, and would be categorised as medium-sized lymphocytes.

Relationships with columnar epithelial cells. There were no desmosomes or other adhesion specialisations between IE lymphocytes and adjacent epithelial cells (Figs. 4.1, 4.2 and 4.4). The lymphocytes lay between the columnar cells rather than within them, but their intrusion into an otherwise orderly epithelium caused them to indent their neighbours (Fig. 4.5). In all instances where the orientation of the cell membranes allowed clear definition of the contact relationships, there was no evidence of cytoplasmic continuity or of breakdown either of the lymphocyte or adjacent epithelial cell membrane. We have not encountered any intraepithelial cell which was unequivocally surrounded by the cytoplasm of a single columnar cell. Depending on the plane of section, parts of two, three or more epithelial cells were seen to make contact with the lymphocyte, proving a truly intercellular situation. At times

lymphocytes/

lymphocytes lay free in pre-existing intercellular spaces, but more commonly they lay close to the columnar cells with an interspace of only 10 to 20 nm, similar in dimensions to the normal epithelial contact gap (Fig. 4.3).

Degeneration. We found no evidence of a significant group of degenerating lymphocytes in the epithelium. Rarely there was encountered an intracellular vacuole containing dense debris, but these had no specific features which might allow them to be identified as degenerating lymphocytes.

Relationships with basal lamina. Lymphocytes were frequently seen crossing the epithelial basal lamina, both in normal and coeliac biopsies. Light and electron microscopy showed constrictions at the level of the interface between epithelium and lamina propria (Figs. 4.4 and 4.6). The basal lamina was breached and its ruptured edge lay close to the margin of the lymphocyte. At times the lymphocyte was accompanied by a protrusion of the base of the epithelial cell through the defect in the lamina (Fig. 4.4). Elsewhere there were similar herniations of the epithelial cells without an accompanying lymphocyte, at least in the plane of section examined (Fig. 4.7). These epithelial herniations ranged from minute tongues to extensive blebs which could contain endoplasmic reticulum and mitochondriae. The protrusion, flanked by the ruptured basal lamina, lay/

lay in close relationship to the components of the lamina propria.

Lymphocyte morphology related to diagnosis. The range of structural variability in the IE lymphocyte population was very wide and there was no significant or consistent difference in lymphocyte structure in the different groups of patients in this study. In general, however, lymphocytes were more numerous and more irregular in outline in the abnormal epithelium of patients with coeliac disease.

Other intraepithelial cells. Occasional IE eosinophils were in the same general relationship to the epithelial cells as the lymphocytes described above. They had typical light and ultrastructural features of eosinophils, including bilobed nucleus, and characteristic compound inclusions with crystalline components. The cell outlines were not amoeboid. IE mast cells were rounded in outline, with small surface projections folded flat by pressure from adjacent cells. The cytoplasm contained typical granules, but material corresponding to discharged granules was not seen near these cells.

Quantitation of IE lymphocytes of human jejunum

This was carried out in 200 biopsies, and values expressed as IE lymphocytes per 100 epithelial cells. Results for 160 biopsies from non-coeliac patients are illustrated in Fig. 4.8. Fig. 4.9 shows/

shows the values in 40 'normal' patients, 20 coeliac patients taking a gluten-containing diet and 20 coeliac patients taking a gluten-free diet. A "normal range" of values was estimated in two ways: from the counts in a group of healthy patients (no small bowel or immunological disease) and from probability curves constructed from the results of counts in the 160 non-coeliac biopsies.

Normal range (from normal patients). Within the group of 200 biopsies there were specimens from 40 patients in whom, finally, no evidence of inflammatory bowel disease or immunological abnormality had been detected. Clinical diagnoses are listed in Table 4.1. The range of IE lymphocyte counts in these 40 biopsies was 9.6 - 39.3; mean 21.1 and SD 7.5. This gives a normal range (mean± 2 SD) of 6.1 - 36.1 IE lymphocytes per 100 epithelial cells.

Normal range from probability curve (Hoffmann, 1963). This is a simple method for the determination of normal values for a clinical laboratory test, based on analysis of routinely submitted samples. The IE lymphocyte counts for the 160 non-coeliac biopsies were arranged in groups with counts of <4; 4.1-8; 8.1 - 12... etc. A cumulative percentage of biopsies for each lymphocyte count value was calculated, and cumulative percent was plotted against IE lymphocyte count value on probability graph/

graph paper. Hoffmann has shown that a straight line drawn through the central points corresponds to a normally distributed series of values and is likely to represent the range of values in a healthy population. Distortions at the upper and lower ends of the curve are due to the presence, in the series, of pathologically high and low values. The range of normal values is obtained by noting where the straight line intersects the 5% and 95% lines of the probability paper. The curve for 160 IE lymphocyte counts is shown in Fig. 4.9; from this a "normal range" of 7-40 is obtained.

Since these two methods give comparable results, the normal range of values for human jejunal IE lymphocyte counts has been taken as 6-40 IE lymphocytes per 100 epithelial cells.

Abnormal IE lymphocyte counts in jejunal biopsies

When a normal range had been established, the results of IE lymphocyte counts in the 200 biopsies were reviewed, to find if any disease was associated with high or low counts, and to review the diagnoses of patients with exceptionally high or low counts.

Adult coeliac disease (Fig. 4.9). Counts in untreated coeliac patients were significantly higher than in the normal group ($P < 0.001$); the values fell significantly ($P < 0.001$) in the group of/

of treated coeliac patients, but not to normal levels. The IE lymphocyte count bore no consistent relation to the clinical state of the patient at the time of biopsy, and the effect of a gluten-free diet on IE lymphocyte count seemed to be relatively independent of other manifestations of histological improvement.

Other gastrointestinal diseases. IE lymphocyte counts were normal in biopsies from patients with ulcerative colitis, Crohn's disease, Whipple's disease, colonic histiocytosis and round-worm infestation.

Autoimmune diseases. Three of 19 patients studied had IE lymphocyte counts above the normal range. Diagnoses were rheumatoid arthritis; Hashimoto's thyroiditis and lupus erythematosis.

Patients with moderately high IE lymphocyte counts. The distribution of 160 counts in non-coeliac biopsies (Fig. 4.8) shows that most fall within the normal range of 6-40, but there is a secondary peak between 38 and 56. This is objective evidence that there is a moderate increase in the numbers of IE lymphocytes in some otherwise normal biopsies, often reported by pathologists as "increased chronic inflammatory cell infiltrate".

Patients with very high IE lymphocyte counts. Details of the 7 non-coeliac patients with high counts are given in Table 4.2.

In/

In case 1, the villi with increased lymphocyte count were overlying lymphoid nodules. Case 6 was the only one of three Indian or Pakistani patients in the series who had biopsy findings which would have been considered abnormal in a Caucasian. Dissecting microscopy showed a ridged mucosa; there were many inflammatory cells in the lamina propria, and the epithelial cell height was reduced. However, these are normal findings in jejunal biopsies from Indians (Baker et al, 1962), and it is not known why the small intestinal mucosa of healthy Indians appears so chronically inflamed. Cases 3 and 5 were suffering from well established immunological diseases, and presumably the increased numbers of gut lymphocytes were another manifestation of the immunological disturbances in erythema nodosum and disseminated lupus erythematosis. The finding of abnormal lymphocyte counts in patients 2, 4 and 7 is very interesting for no satisfactory diagnosis had been made in these patients despite the presence of very real and incapacitating symptoms - chronic diarrhoea, sore tongue, and abdominal pain. Certainly in case 7 it is likely that the increased number of gut lymphocytes was related to the underlying disease process, for spontaneous remission of symptoms (on no specific treatment) was accompanied by a fall in the IE lymphocyte count.

Patient with low IE lymphocyte count. In one 57 year old man, the count was only 5.8 lymphocytes per 100 epithelial cells. This man/

man had chronic glomerulonephritis of moderate severity and had been given a six-month course of azathioprine. While taking the drug he developed diarrhoea, which persisted after treatment had been stopped. Intestinal biopsy was carried out six months later and the histology had been considered to be normal. As all other investigations had proved negative a diagnosis of "irritable bowel syndrome" had been made. It seems likely that the azathioprine treatment was the cause of the depletion of IE lymphocytes and possibly also of the chronic diarrhoea.

Conclusions

In their light microscopic and ultrastructural features, the human IE lymphocytes resemble their counterparts in the intestines of other species (as described in Chapter 2). The IE lymphocytes are basally situated in the epithelium, they often have amoeboid outlines; they quite definitely lie between and not within the epithelial cells; and I have found no evidence that lymphocytes degenerate within the epithelium. Frequently, lymphocytes can be seen crossing the basal lamina but there are no features to indicate the direction of movement. In addition, a hitherto undescribed phenomenon has been described - the presence of epithelial herniations through the basal lamina. It is likely that these are associated with the passage of lymphoid cells across the basal lamina.

It proved relatively easy to quantitate the IE lymphocytes in routinely processed jejunal biopsies. The normal range of values has been established as 6-40 IE lymphocytes per 100 villous epithelial cells. Only in coeliac disease were the IE lymphocyte counts consistently higher than normal and the relevance of this finding is reinforced by the fact that the counts fell when patients were treated with a gluten-free diet.

Table 4.1 Final diagnoses in the 40 patients used to establish the normal range of IE lymphocyte counts

Diagnosis	Number of Patients
Relatives of patients with disaccharidase deficiency	7
Primary adult disaccharidase deficiency	3
Paget's disease	2
Idiopathic hypercalcuria	3
Diabetes mellitus	1
Schizophrenia (to have trial of gluten withdrawal)	4
Haemochromatosis	3
Small stature	3
Nutritional vitamin or iron deficiency (after therapy)	8
Orthostatic proteinuria	1
Investigation for possible mesenteric ischaemia (no small bowel disease found)	5
Total	40

(21 male, 19 female; aged 11-76)

Table 4.2 Clinical details of seven patients with very high IE lymphocyte counts

Case Number	IE lymphocyte count*	Age (years)	Sex	Clinical Features
1	62.8	18	Male	Blind loop syndrome with B_{12} deficiency. Jejunal biopsy was of a lymphoid nodule. Subsequent biopsy normal with IE lymphocyte count of 29
2	67.2	54	Male	Chronic diarrhoea and rectal mucous discharge; no cause found
3	71.8	32	Female	Systemic lupus erythematosis - 3 years on oral steroid therapy
4	74.3	33	Female	Persistent sore tongue; no cause found
5	80.7	26	Female	Erythema nodosum. Patient volunteered as normal control for disaccharidase assay
6	96.7	31	Male	Indian doctor with rickets and diarrhoea; lactase deficiency
7	150.1	54	Female	Very severe intermittent abdominal pain; no cause found; spontaneous remission; IE lymphocyte count one year later was 26

* Expressed as IE lymphocyte count per 100 villous epithelial cells.

Fig. 4.1 IE lymphocyte situated basally between crypt cells. A nucleolus, and several lysosome-like inclusions can be seen. LP-connective tissue of the lamina propria. x 28,000

Fig. 4.2 Compact round IE lymphocyte with prominent nucleolus. A few lysosome-like bodies and a small multivesicular body are present in the cytoplasm. x 38,000

Fig. 4.3 IE lymphocyte showing compact Golgi apparatus, mitochondria and scattered ribosomes. The cell is in contact with surrounding columnar cells and with a second lymphocyte (L). The island of cytoplasm (arrowed) may indicate an invaginated lymphocyte process separated from the parent cell. x 62,000

Fig. 4.4 Migrating lymphocyte, part of which lies outside the epithelium, in contact with the cells of the lamina propria (LP) which include a macrophage (M). The basal lamina is broken to allow passage of the cell and there is a small herniation of the adjacent epithelial cell (arrowed). The irregular pseudopod-like processes of this cell seem to indent adjacent columnar cells. x 30,000

Fig. 4.5 IE lymphocyte with an irregular pseudopodial projection (P) apparently pushing between epithelial cells. There are scattered ribosomes within its pale cytoplasm, contrasting with surrounding denser epithelial cells. The irregular clefts at the lymphocyte surface suggest active expansion at this point. x 45,000

Fig. 4.6 Two IE lymphocytes (L) situated partly within the epithelium and partly in contact with the lamina propria in which a macrophage (M) is seen. The basal lamina is breached at the points arrowed. x 40,000

Fig. 4.7 Base of columnar epithelium showing the ruptured basal lamina (arrowed) with an epithelial herniation projecting into the lamina propria. The pale island of cytoplasm (P) may be a process of a migrating lymphocyte, most of which lies in another plane. x 34,000

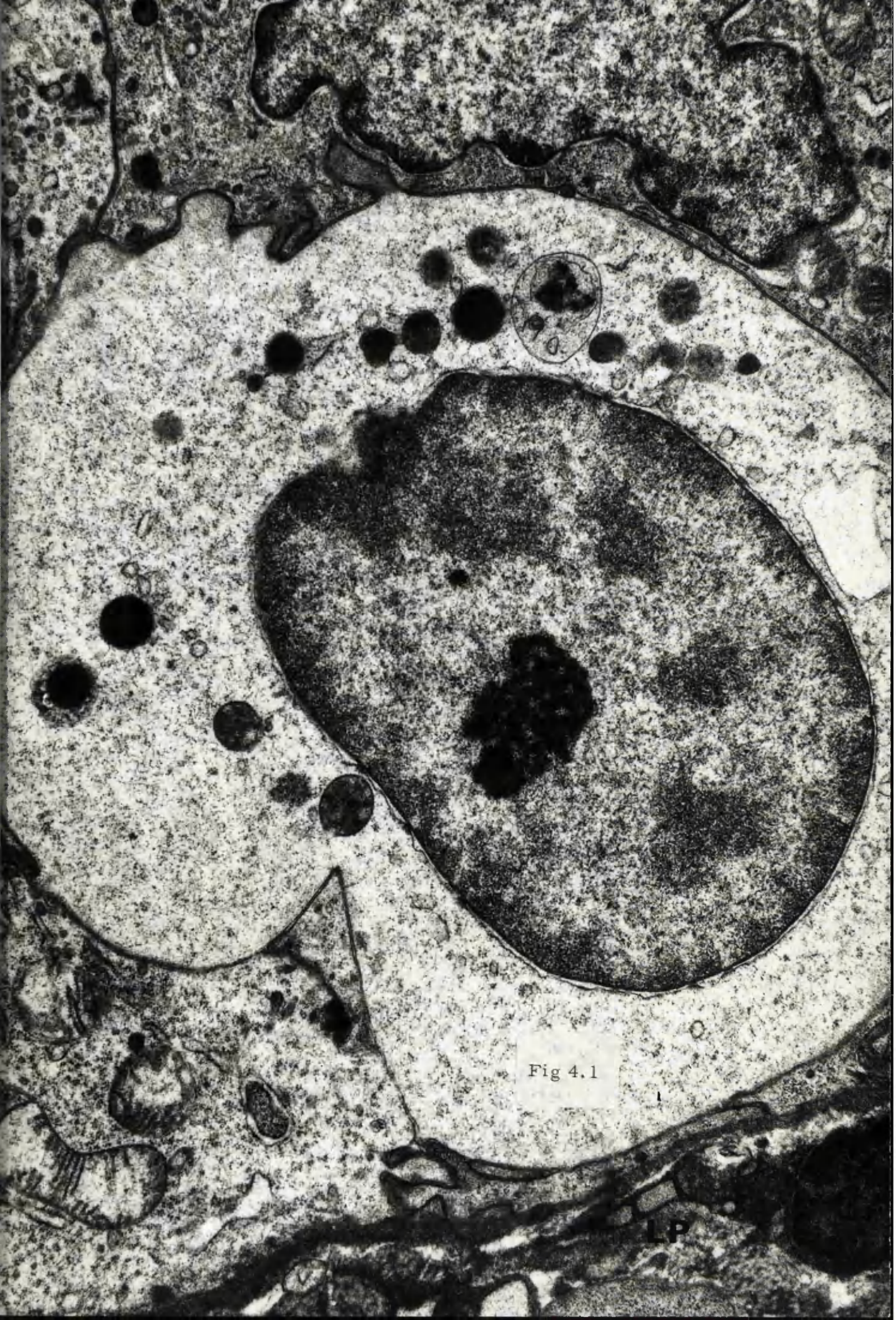

Fig 4.1

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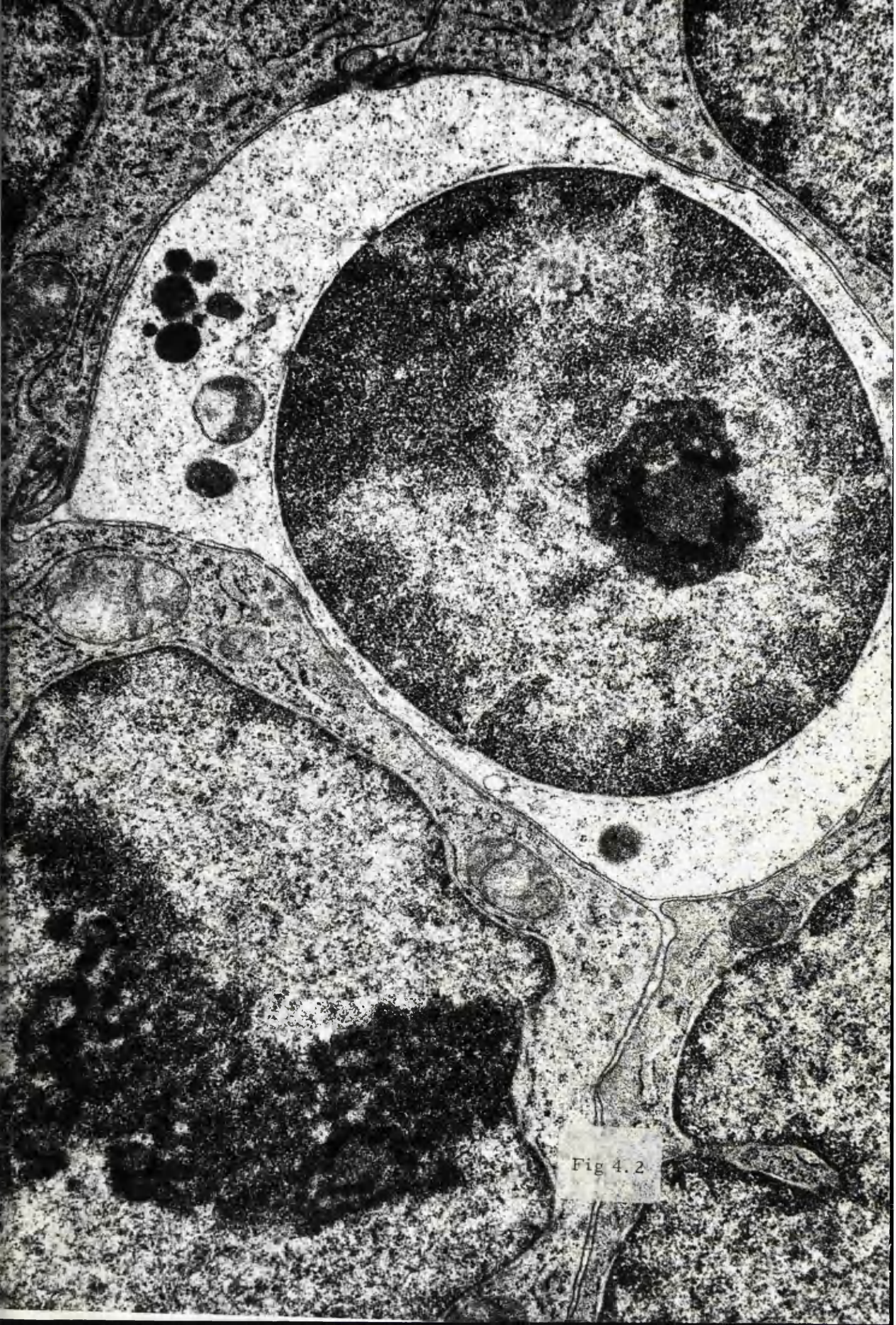

Fig 4.2

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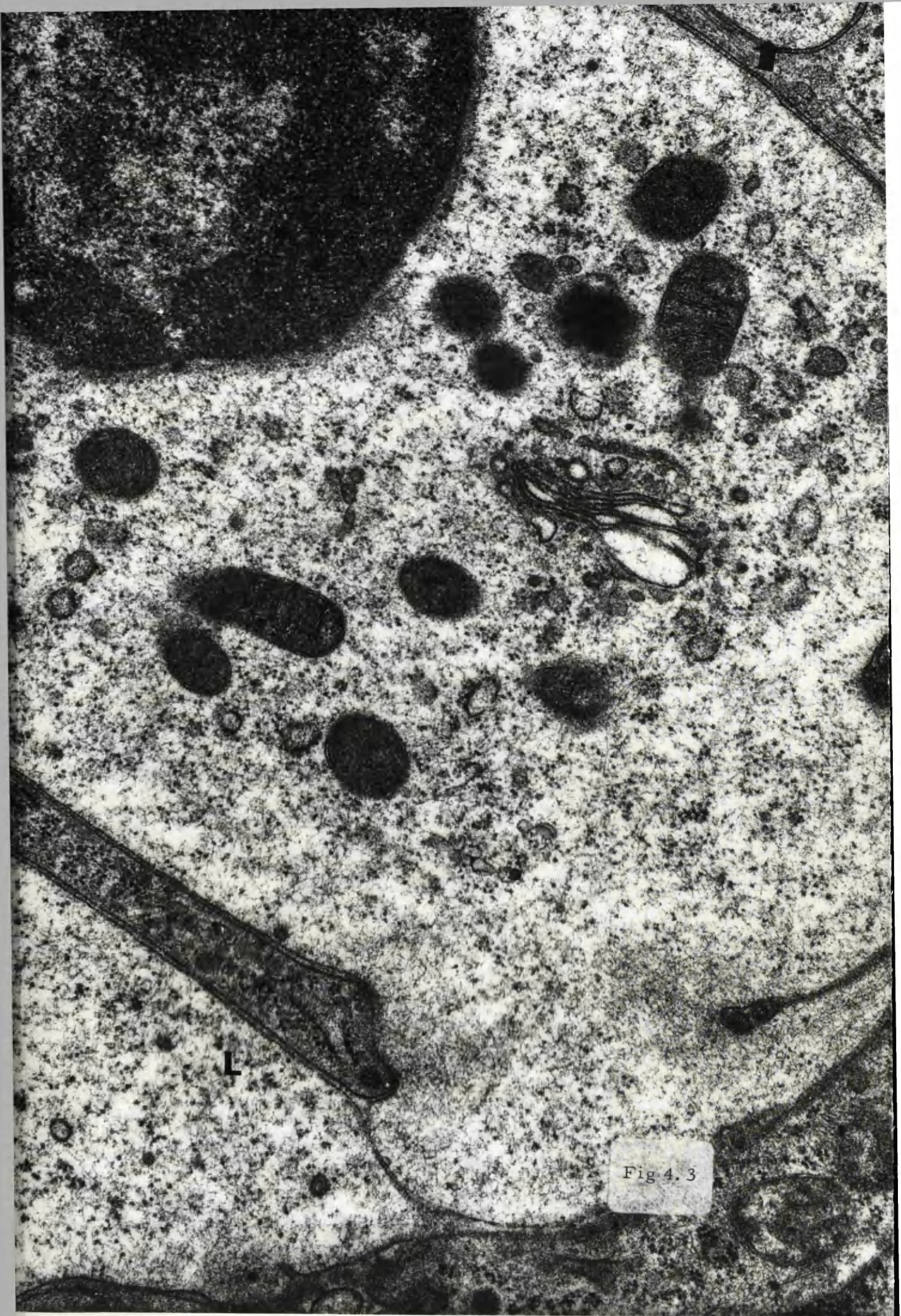

Fig 4.3

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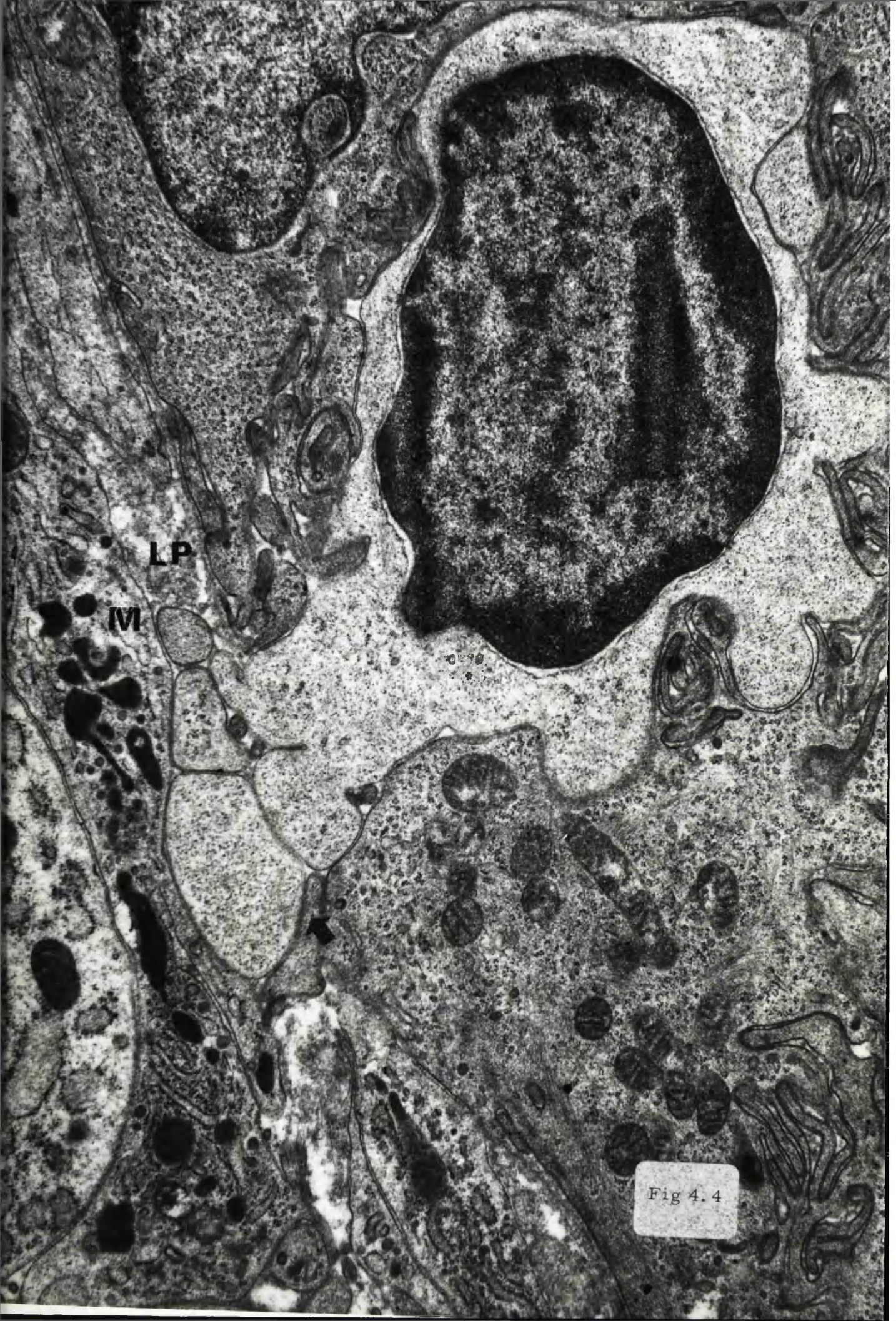

Fig 4.4

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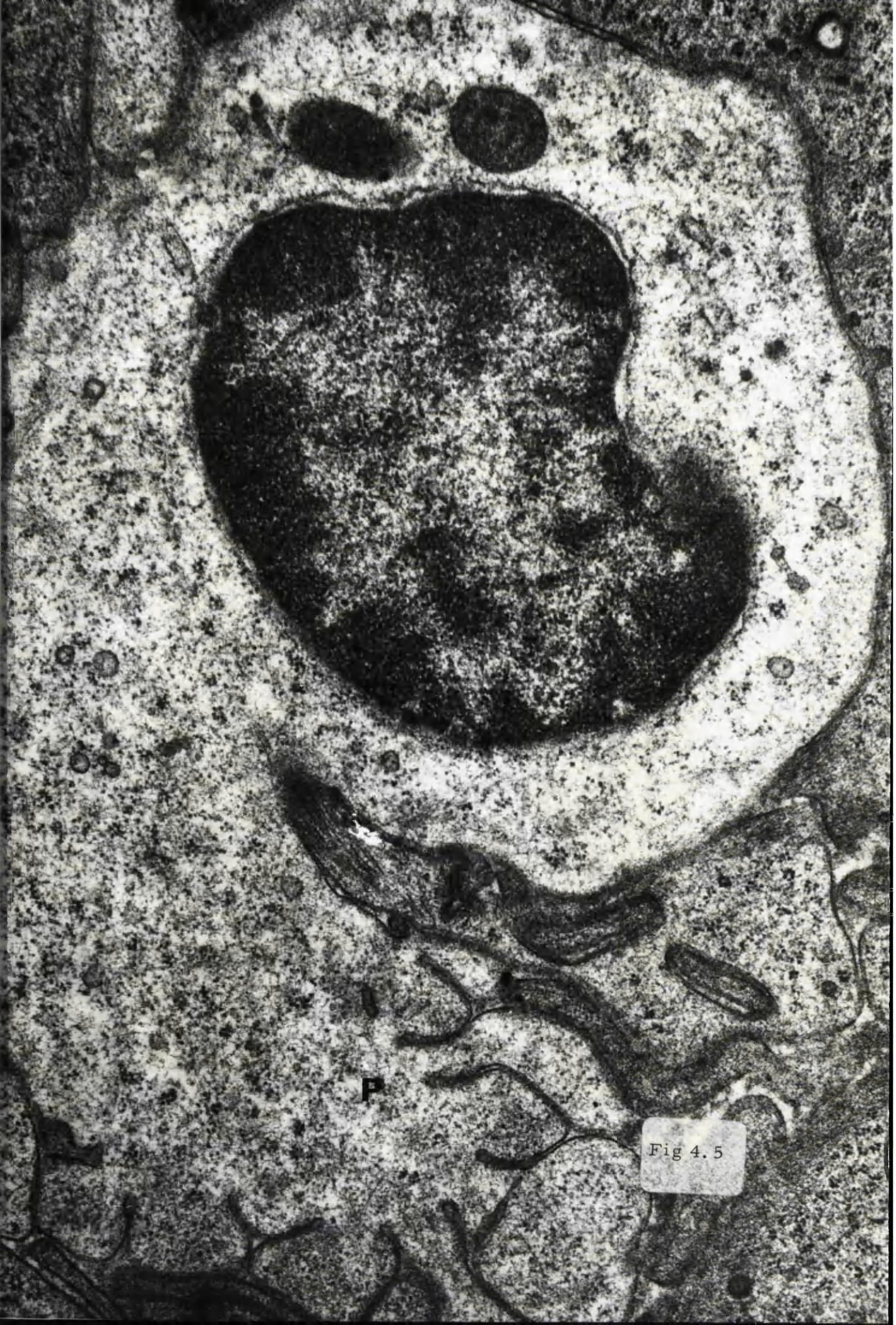

Fig 4.5

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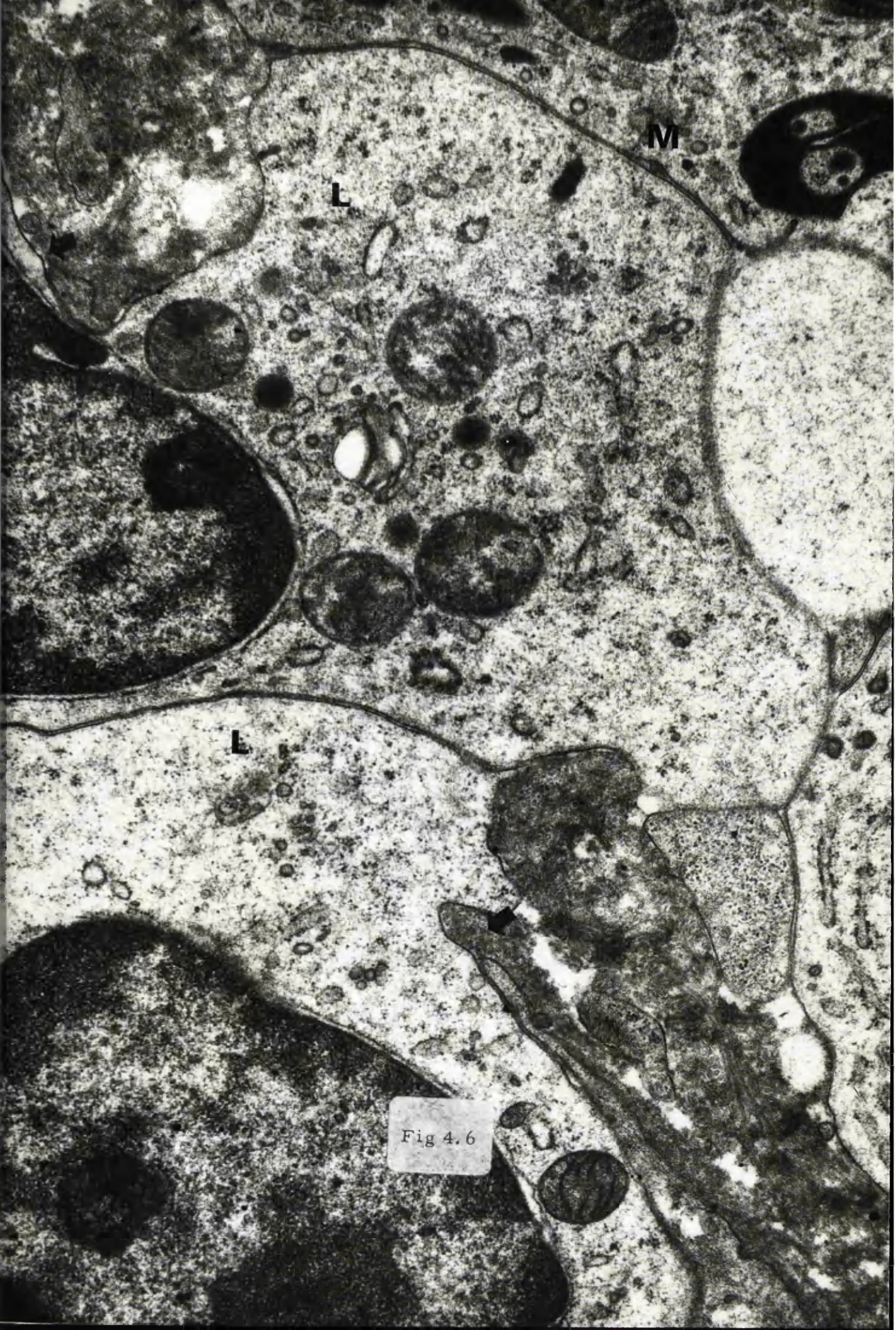

Fig 4.6

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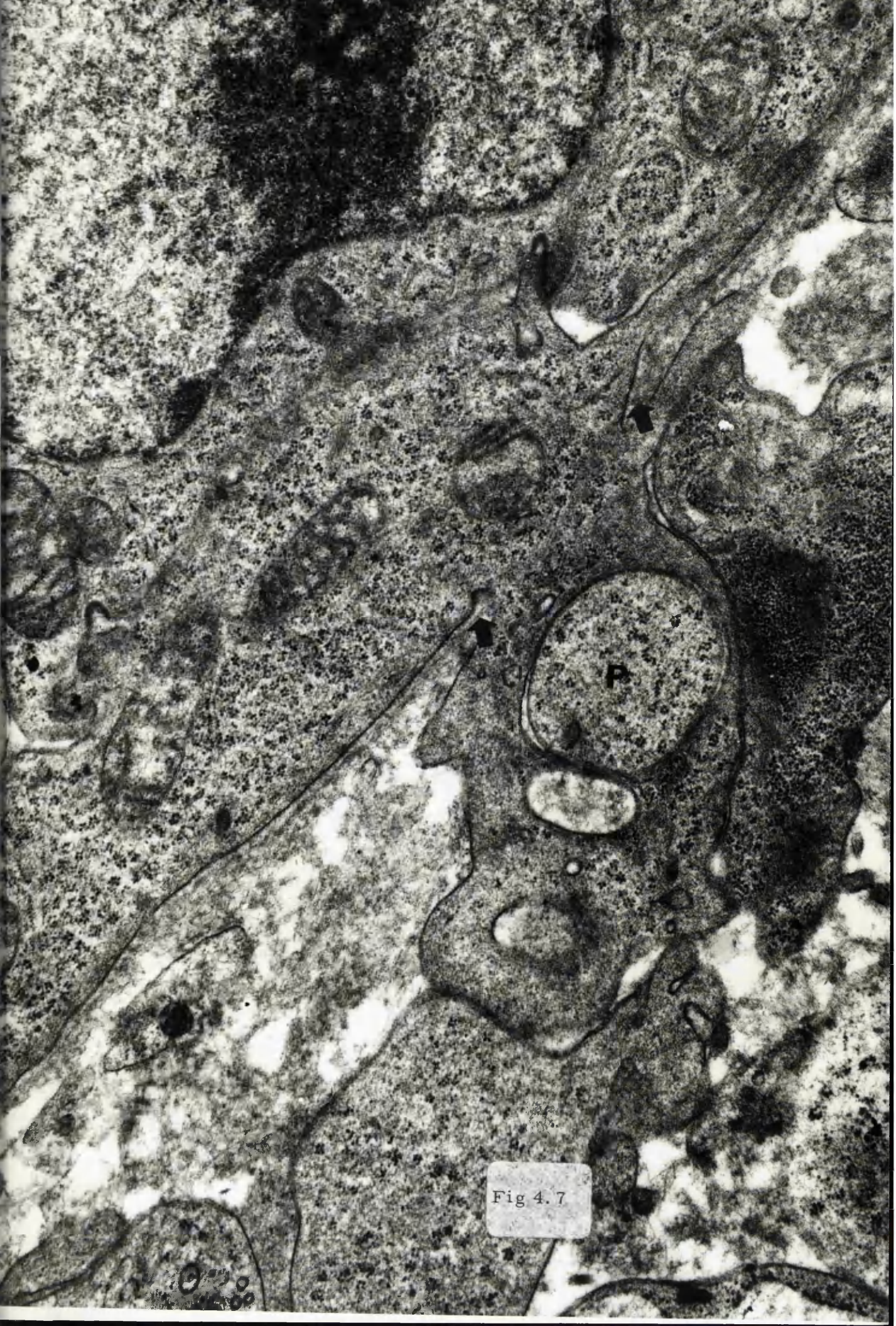

Fig 4.7

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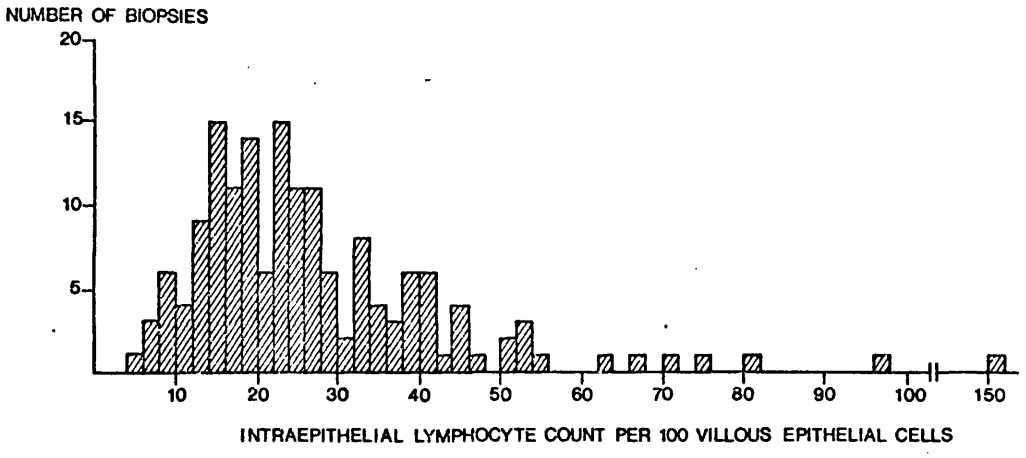

Fig. 4.8 Distribution of IE lymphocyte counts in 160 jejunal biopsies from patients who did not have coeliac disease.

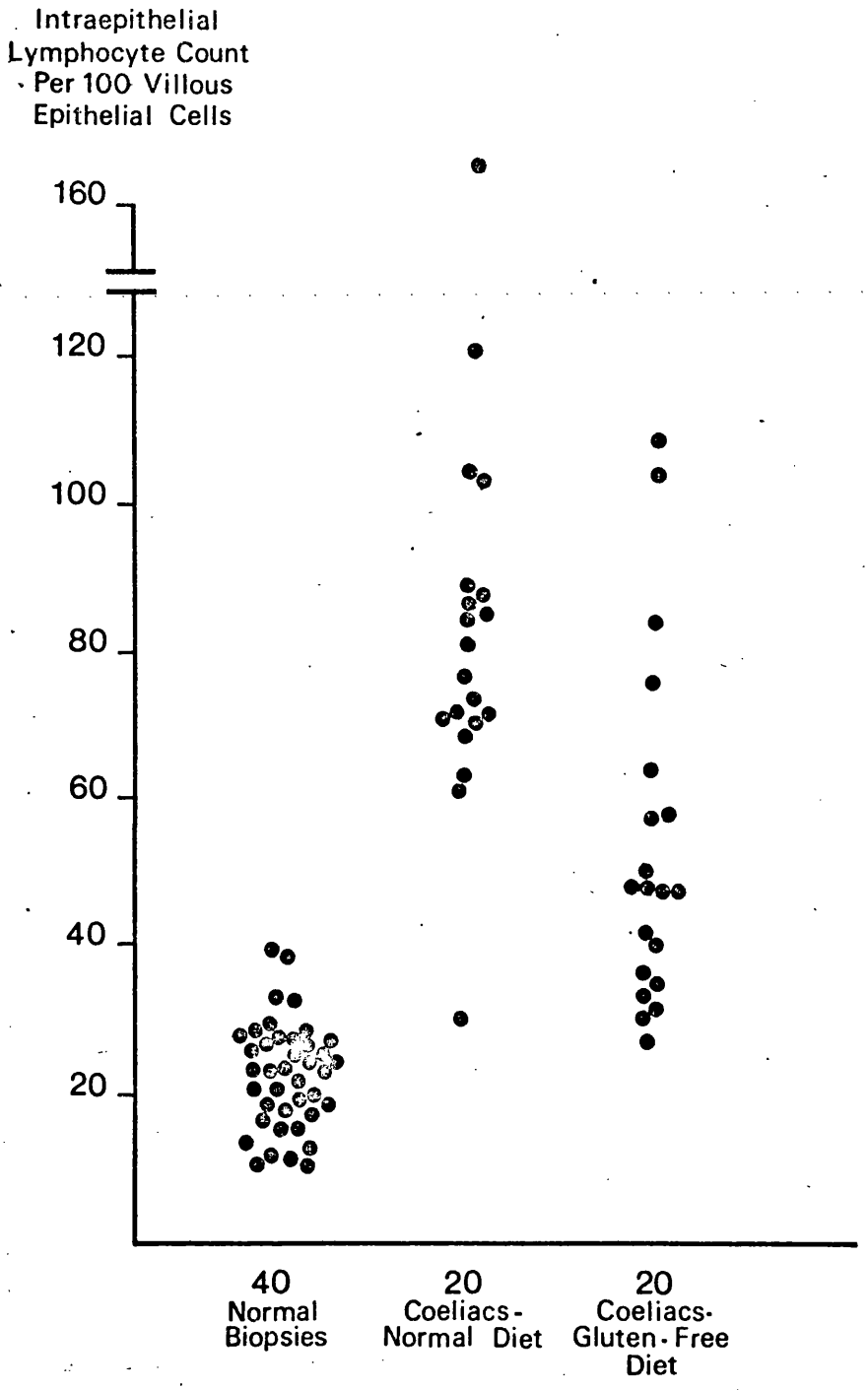

Fig. 4.9 IE lymphocyte counts in jejunal biopsies from 40 immunologically normal patients, 20 adult coeliac patients taking a gluten-containing diet and 20 coeliac patients taking a gluten-free diet.

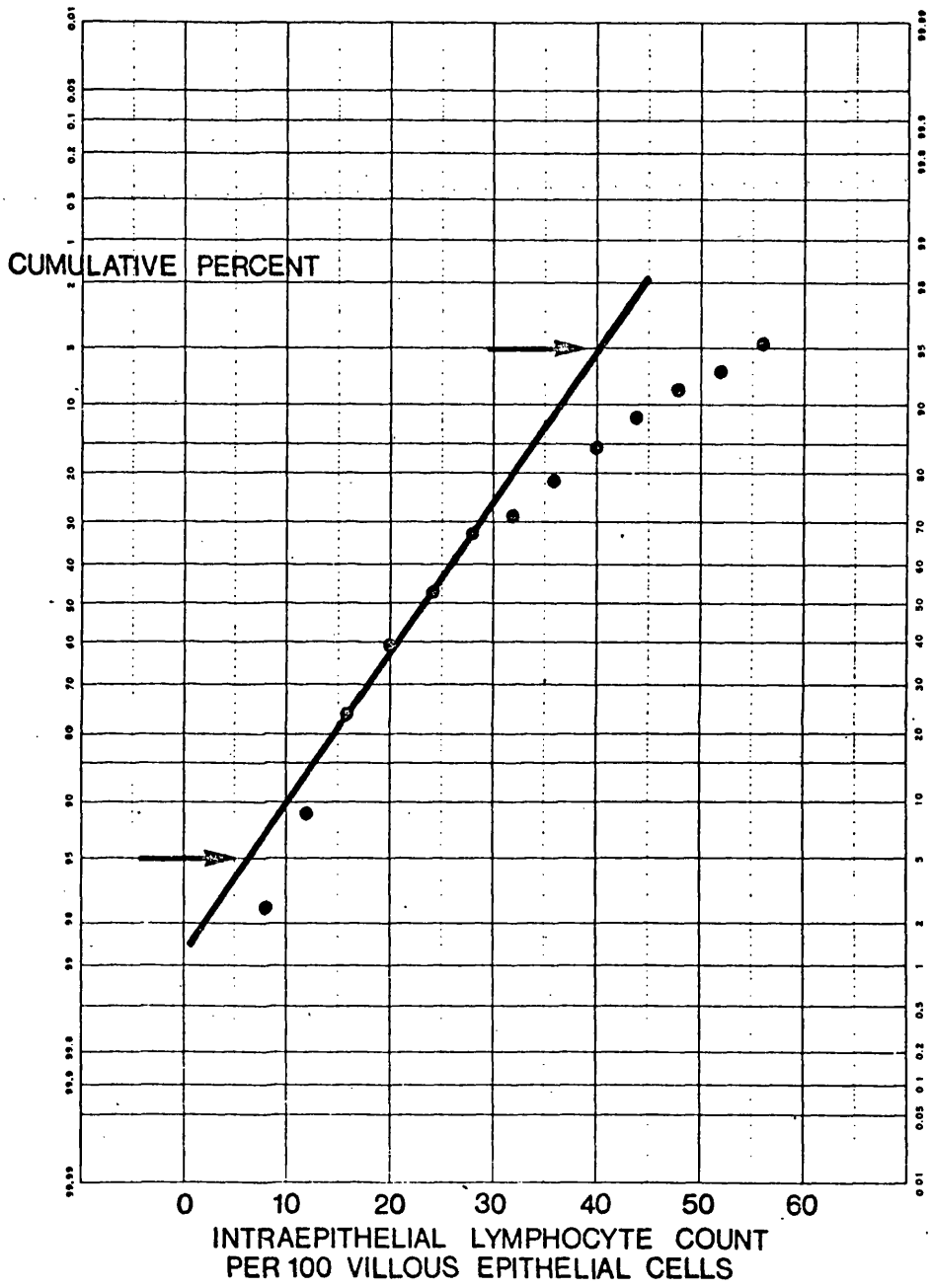

Fig. 4.10 IE lymphocyte count plotted against cumulative percentage of 160 jejunal biopsies from patients who did not have coeliac disease.

Chapter 5

"Antigen-free" grafts of intestine. An experimental model for the study of immune processes in the small intestine.

Several recent studies in germ-free animals have illustrated the value of comparatively antigen-free gut for investigation of intestinal immunology (Crabbe et al, 1968, 1969; Nash et al, 1969; Beneviste et al, 1971). However, germ-free animals are neither completely free from extrinsic antigens in food, nor readily available. Ideally one would prefer to use a completely antigen-free preparation of intestine, but it is only in foetal life that the intestine is in this state, and to maintain it in the adult it would be necessary to isolate a length of intestine before or immediately after birth. Such a procedure is feasible in large animals such as rabbits (Perey and Good, 1968; Stramignoni et al, 1969) but would be impossible in rats and mice because of the narrow calibre of the gut at birth. So I approached the problem of preparing antigen-free gut in an entirely different way.

It is already known that small fragments of rodent tissue will grow and develop when transplanted into a heterotopic site. Intestine has been successfully transplanted into the anterior chamber of the eye (Kammeraad, 1942). Endocrine glands, kidney and thymus survive and grow when implanted into heterotopic sites (Everett, 1956; Pepper, 1961; Stone and Kennedy, 1962; East and Parrott, 1964; Barakat and Harrison, 1971). Allografts of testis, ovary, intestine and spleen, implanted/

implanted heterotopically, take at first, and then are rejected (Buck, 1963). Thus there seemed no reason why grafts of mouse small intestine should not take and grow, especially if the grafts were of sterile foetal intestine, implanted in syngeneic hosts.

I found that when fragments of foetal intestine were grafted under the kidney capsule of adult syngeneic hosts they took well, grew (Fig. 5.1) and developed normally, although similar grafts did not survive when implanted into the peritoneal cavity within a millipore chamber. The technique of graft implantation has been described in Chapter 3. These grafts have been examined in a number of ways: morphologically with the dissecting microscope and by light and electron microscopy; epithelial cell kinetics were investigated by ^{3}H thymidine labelling and autoradiography. Other properties investigated include sterility of grafts, their lymphatic drainage, and secretion of immunoglobulins into the graft lumen.

General experience of grafting technique

When the technique was first used there was some mortality due to the anaesthetic and to bleeding from unnecessarily deep incisions in the renal capsule. However, in the last 200 recipient mice there has been no operative mortality and at the last 50 post-mortem examinations (with bilateral grafts in all animals) 98 established grafts have been found. It is interesting to note that although infection has not occurred when foetal donor tissue is used, on the few occasions when donor intestine was taken from postnatal mice (8 days old) some grafts were clearly infected and histological examination showed abscess formation.

Growth and development of normally sited small intestinal mucosa

This description is based on examination of intestinal histology from 86 CBA mice and 42 BALB-c mice, aged from 1 day to 8 months.

In newborn mice the small intestine is very immature, with low villi and no well-defined crypts of Lieberkuhn. By 3 days an orderly pattern of crypts and villi has been established with mitosis taking place in the crypts and newly formed cells passing/

passing from the crypts up the sides of the villi. Paneth cells and goblet cells are present soon after birth, but lymphoid cells do not appear in the mucosa until the third week of life, around the time of weaning. Tiny Peyer's patches are present at birth; these enlarge during the first month of life and germinal centres appear from the fifth week. Thereafter there are no further changes in general morphology.

Growth and development of isografts of small intestine implanted under the kidney capsule

This description is based on studies of 190 isografts of small intestine in CBA mice and 52 grafts in BALB-c mice. Grafts obtained from 1 day to 8 months after implantation were examined histologically. Dissecting microscopy was carried out on 16 grafts, and electron microscopy on 12.

Immediately after implantation some grafts had small areas of haemorrhage (presumably due to handling) but there was no evidence of disintegration of the tissues. 4 or 5 days after implantation, villi and crypts formed, and Paneth and goblet cells appeared. Later blood vessels and lacteals could be identified and smooth muscle developed below the mucosa. There were aggregates of lymphocytes, resembling immature Peyer's/

Peyer's patches, but without germinal centres. Epithelial cell mitosis was confined to the crypts, and epithelial cells were extruded from the tips of villi, accumulating as debris in the graft lumen. This debris could be washed away to reveal a completely normal appearance of villi as judged by examination with the dissecting microscope (Fig. 5.2). About 18 days after grafting, lymphocytes and plasma cells appeared in the loose connective tissue of the lamina propria and some IE lymphocytes were present.

Grafts maintained essentially normal histological appearances up to 8 months (the longest time studied) and duodenum (with Brunner's glands) jejunum (with tall villi and short crypts) and ileum (with shorter villi and many goblet cells) could all be recognised from their general morphological features. Fig. 5.3 illustrates the typical histological appearance of an established graft. Figs. 5.4 to 5.7 illustrate the normal ultrastructural appearances of grafts.

Growth and development of isografts of small intestine in millipore chambers within the peritoneal cavity

17 grafts were implanted in CBA mice, within millipore chambers, and recipients were killed 3-18 days later. The grafts were examined with the dissecting microscope and by routine histological/

histological methods. Examination of the contents of the millipore chambers showed that none of the grafts had enlarged. With the dissecting microscope, recognisable "villi" were present in only 4 of the 17 grafts; all the other chambers contained debris. Histological examination confirmed that the millipore chambers had remained intact throughout the experiment, for there was no infiltration of host cells. The areas that had been considered to have "villi" were found, histologically, to consist only of the connective tissue cores of submucosa and villi (Fig. 5.8).

Growth and development of isografts of colon implanted under the kidney capsule

6 grafts of colon were examined 15-60 days after implantation. They also grew and developed normally, but the colonic mucosal cells secreted mucus into the lumen so that the grafts enlarged and became cystic swellings, sometimes as large as the kidney itself. However, as can be seen from Fig. 5.9, the morphology of the grafted colonic mucosa was completely normal.

Growth and development of isografts of small intestine in rats

60 isografts of small intestine were implanted in Wistar and SA rats, and examined 3-35 days later. These grafts also took and grew/

grew well, and appeared normal histologically (Fig. 5.10).

Isografts of small intestine - lymphatic drainage

Carbon was injected into the renal subcapsular space (adjacent to the grafts) in 4 mice (2 left-sided graft and 2 right-sided). 24 hours later, carbon could be clearly identified in the tiny lymph node which lay anterior to the renal artery, beside the aorta (on the left or right side, according to the location of the injection). In one of the mice, carbon was also present in lymph nodes of the mediastinum.

Tests for bacterial contamination of grafts

Bacteria were never seen in the electron microscopic studies of grafts. In addition, when 4 grafts were cultured aerobically and anaerobically for 72 hours, no growth was obtained.

Epithelial cell proliferation in grafts of small intestine

38 days after implantation of grafts, 4 mice were given 100μc ^{3}H-thymidine intraperitoneally. 2 were killed 2 hours later, and the others at 24 hours. Sections of the animals' own intestine, and the grafts, were processed for autoradiography as described in Chapter 3. Examination of the autoradiographs confirmed/

confirmed that epithelial cell mitosis was confined to crypt cells (Fig. 5.11) and that 24 hours later the leading edge of the column of labelled cells had moved to the basal part of the villous layer (Fig. 5.12). However, when the autoradiographs of the grafts were compared with the autoradiographs of the host mouse's normally sited intestine two differences were apparent. At 2 hours the dividing crypt cells in the grafts were less heavily labelled than the crypt cells in the host intestine. Also, at 24 hours the leading edge of labelled cells in the grafts had reached only the basal third of the villous layer, whereas in the normally sited intestine it was more than halfway up the villi.

Immunoglobulin content of grafts

9 mice, which had had bilaterial grafts of small intestine implanted 6 months previously, were studied. Immunoglobulins were assayed by radial immunodiffusion in serum, and in saline extracts of tissues (100 mg/ml PBS) as described in Chapter 2. The results are summarised in Table 5.1. It can be seen that IgA was the predominant immunoglobulin in extracts from mesenteric lymph node, normally sited small intestine and grafts, but values were much higher for grafts than for other tissues./

tissues. When the IgA values for graft wall and graft contents are compared, it is clear that most of the IgA is derived from the cheesy luminal debris within the grafts. This recent finding strongly suggests that IgA has been actively secreted into the graft lumen, and merits further investigation.

Conclusions

The technique of grafting foetal mouse intestine has proved to be simple and appropriate to conventional laboratory conditions, and gives a preparation which is sterile and has never been exposed to intraluminal antigens. Grafts of small intestine appear normal when examined by dissecting microscopy, light microscopy and ultrastructurally. They have a normal pattern of epithelial cell mitosis and migration, though the rate of crypt cell mitosis and of cell transit along the villi resemble those of germ-free intestine (Abrams et al, 1963) rather than the host animal's own intestine. Finally, there is evidence that IgA concentration is higher in the graft lumen than in blood, so it is very likely that IgA is secreted into the lumen.

Table 5.1 Immunoglobulins in sera and in saline extracts of mouse tissues (means)

Tissue	Number of specimens	Immunoglobulin content, units/ml G1	G2	A	M
Pooled normal mouse serum	1	100	100	100	100
Experimental mouse serum	9	136	143	153	98
Isografts of SI	18	3.4	4.1	54.1	-
Graft wall	6	2.3	1.7	7.1	-
Graft contents	6	5.0	4.9	58.6	-
Normally sited SI	6	0.5	0.5	5.3	-
Mesenteric LN	9	1.4	1.6	3.8	-
Kidney	3	1.5	1.9	1.3	-

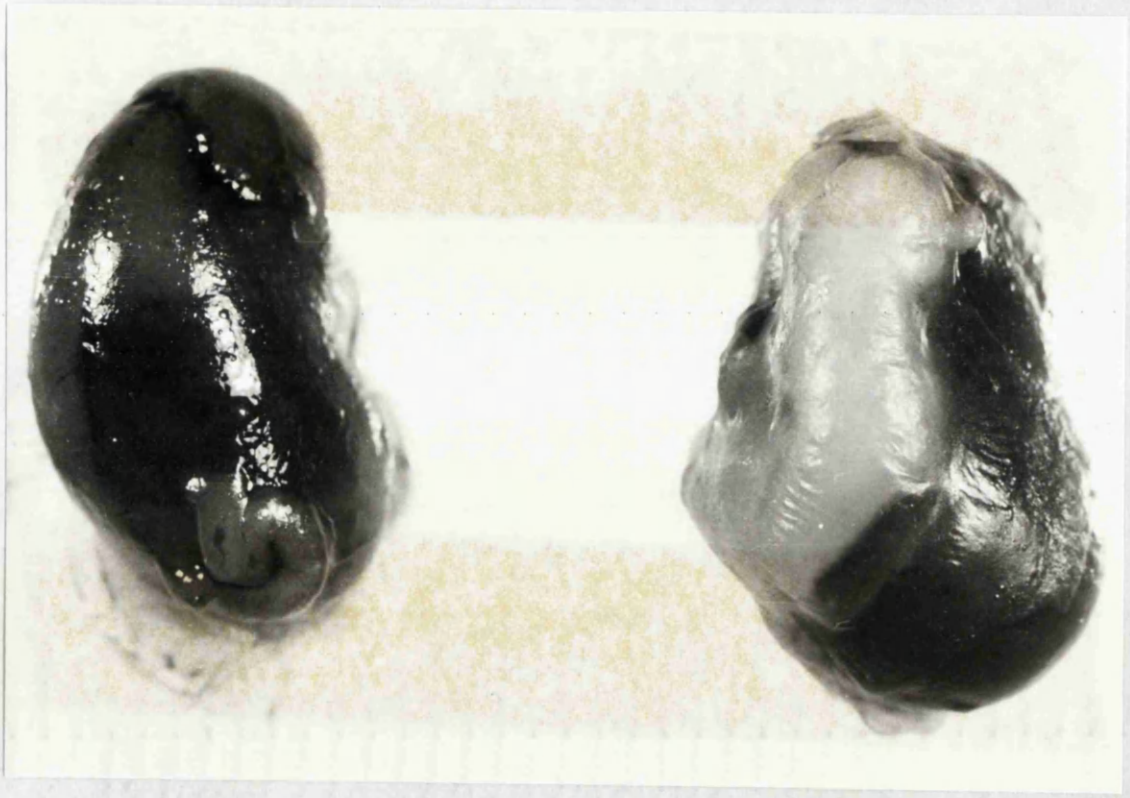

Fig. 5.1 Isografts of foetal small intestine in CBA mice. Left - 1 hour after implantation; right - 80 days after implantation. x c8

Fig. 5.2 Dissection microscope appearance of an isograft of foetal small intestine in a CBA mouse, 38 days after implantation. Some debris from the graft lumen still adheres to the mucosal surface. Crystal violet, x c50

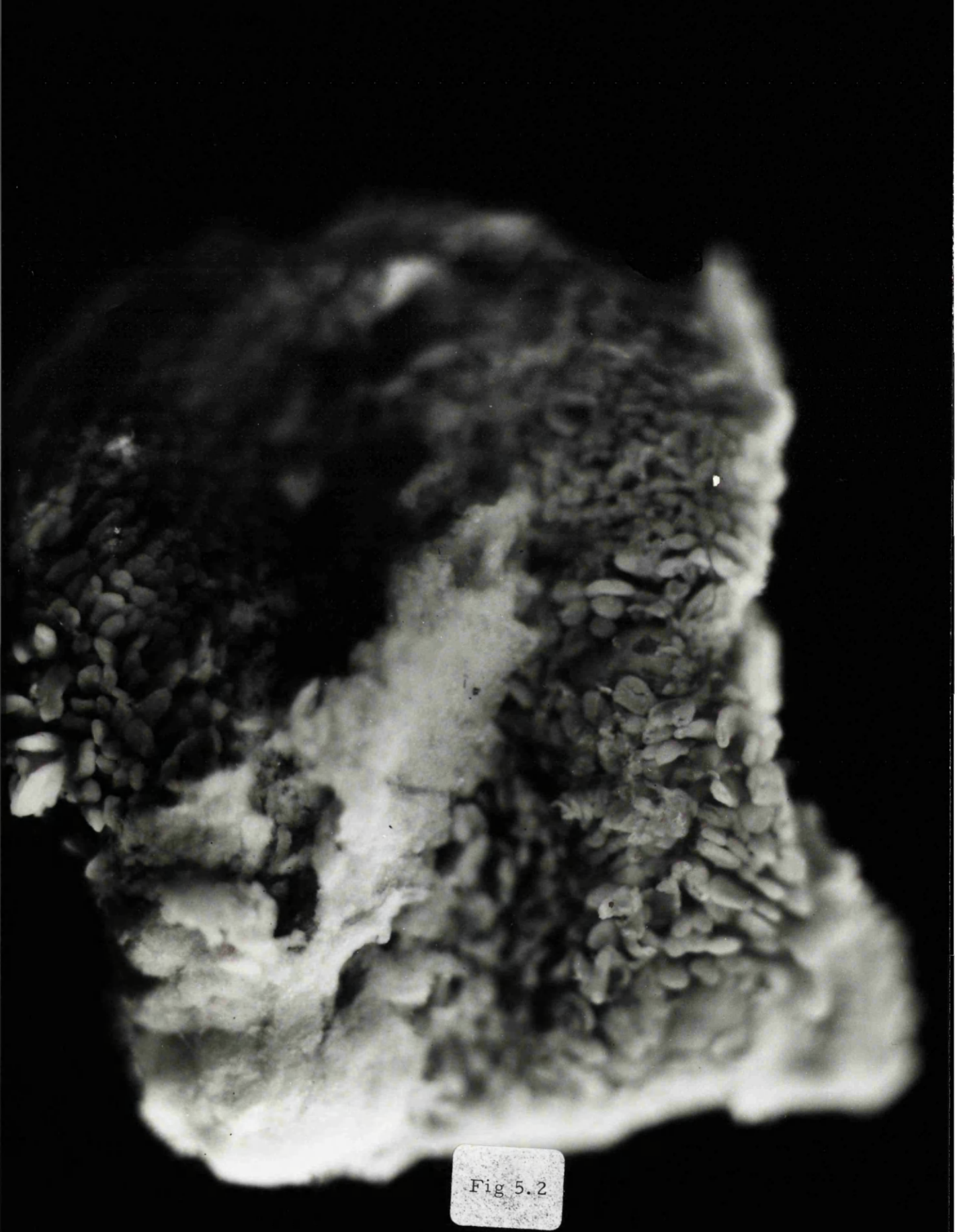

Fig 5.2

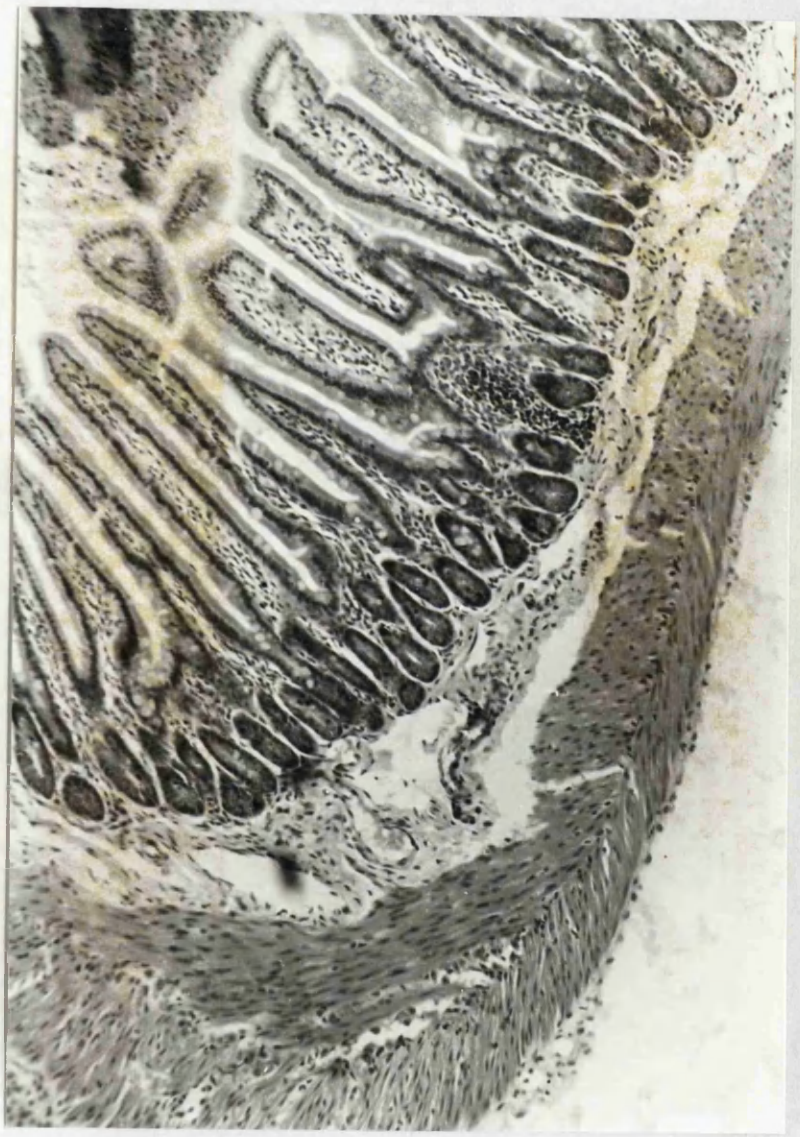

Fig. 5.3 Isograft of foetal small intestine in a BALB-c mouse, 37 days after implantation. H & E x 150

Figs. 5.4 - 5.7 Ultrastructural features of isografts of foetal small intestine in CBA mice.

Fig. 5.4 Completely normal appearance of villous epithelium, illustrating several enterocytes and a goblet cell. x 6000

Fig. 5.5 Section of part of the base of a crypt, with adjacent lamina propria (LP) and blood vessel (B). Parts of several Paneth cells are present. There is a small nerve fibre in the lamina propria. x 6000

Fig. 5.6 Cross sections of several crypts, illustrating crypt chief cells and endocrine cells (E). x 4200

Fig. 5.7 Tangentially cut section of the apical part of an enterocyte, illustrating the brush border and apical pinocytotic vesicles. This tissue is from a graft 14 days after implantation. (In mice, cessation of pinocytosis occurs at around 16 days after birth). x 39000

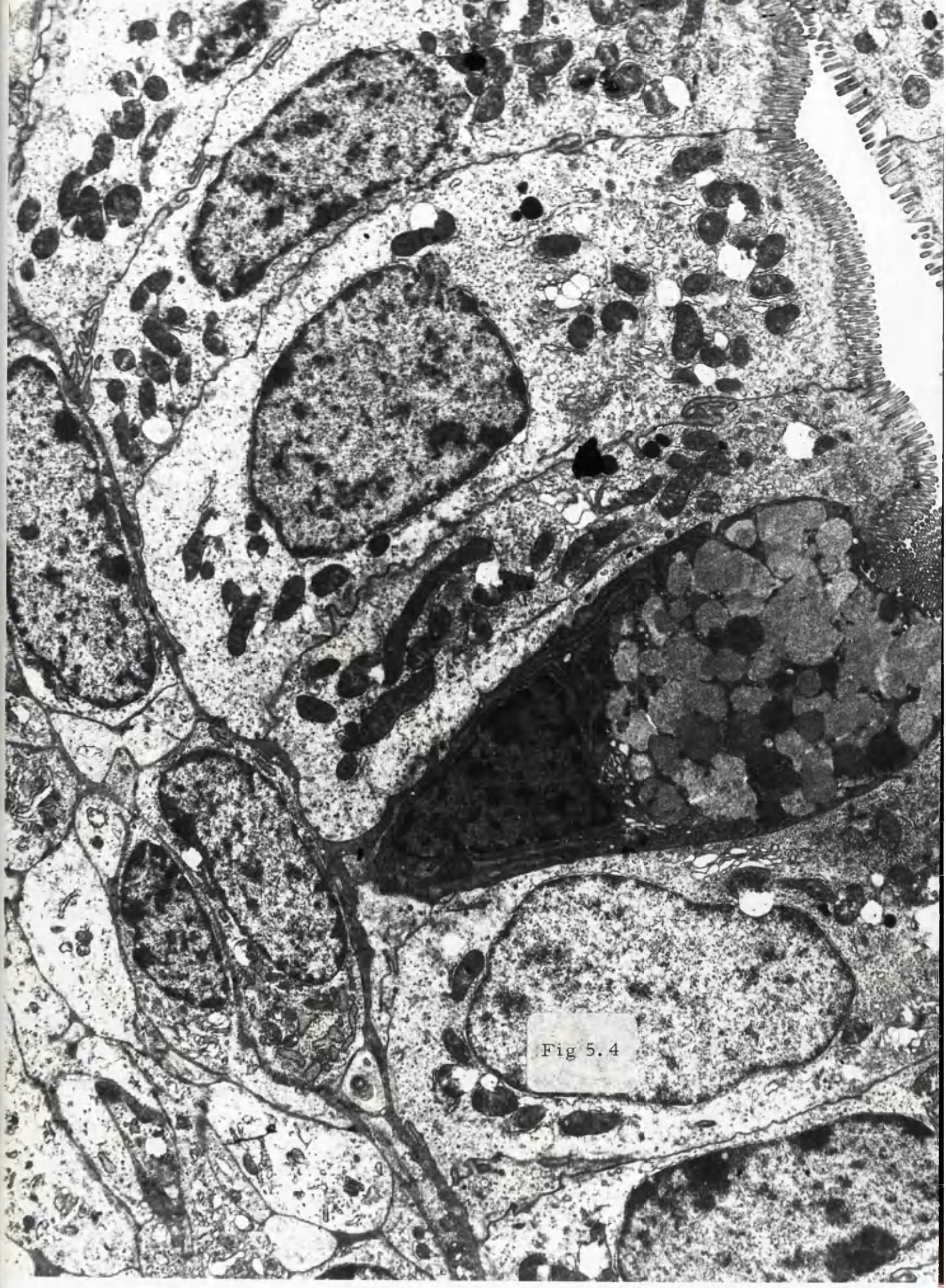

Fig 5.4

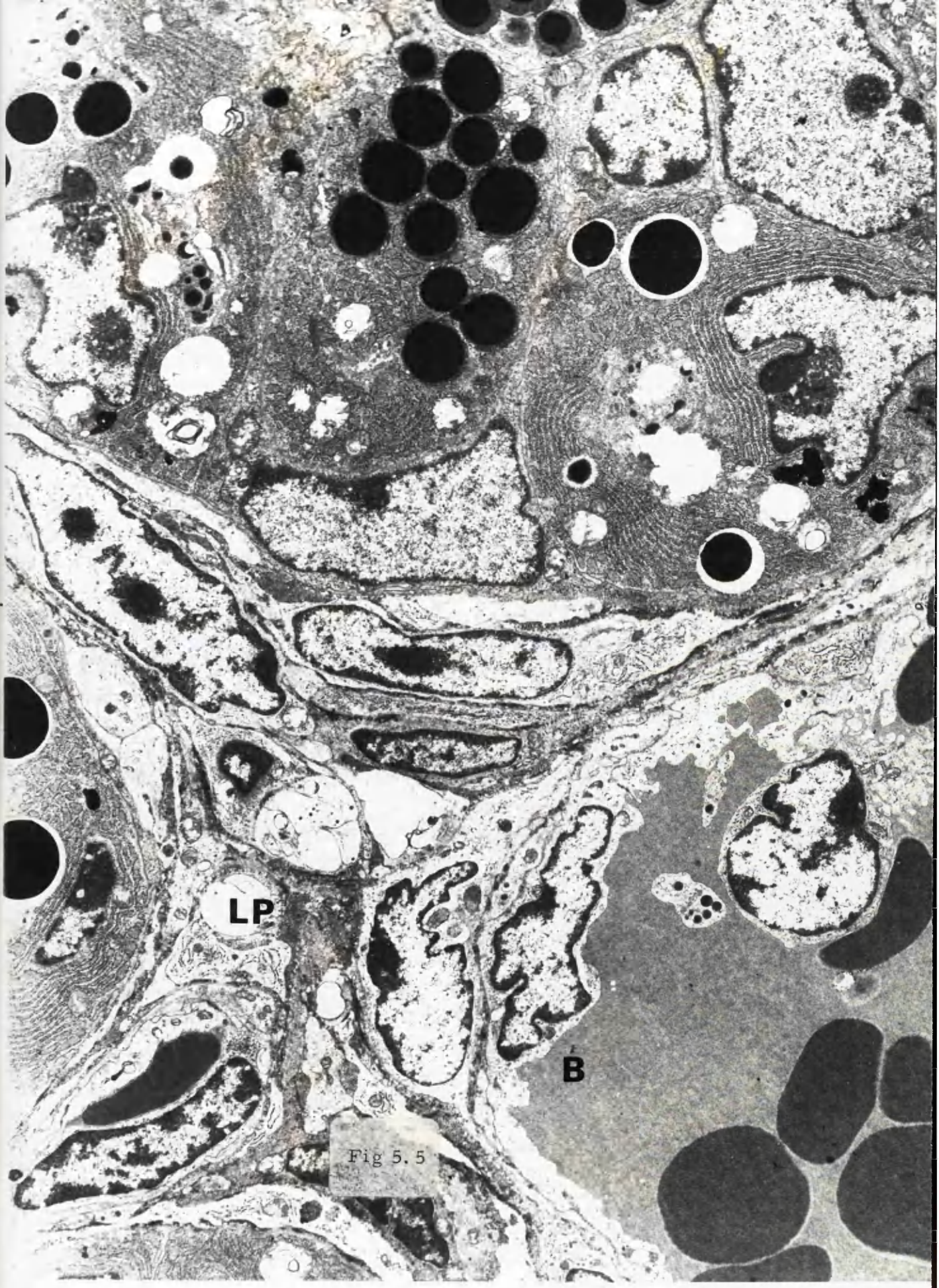

Fig 5.5

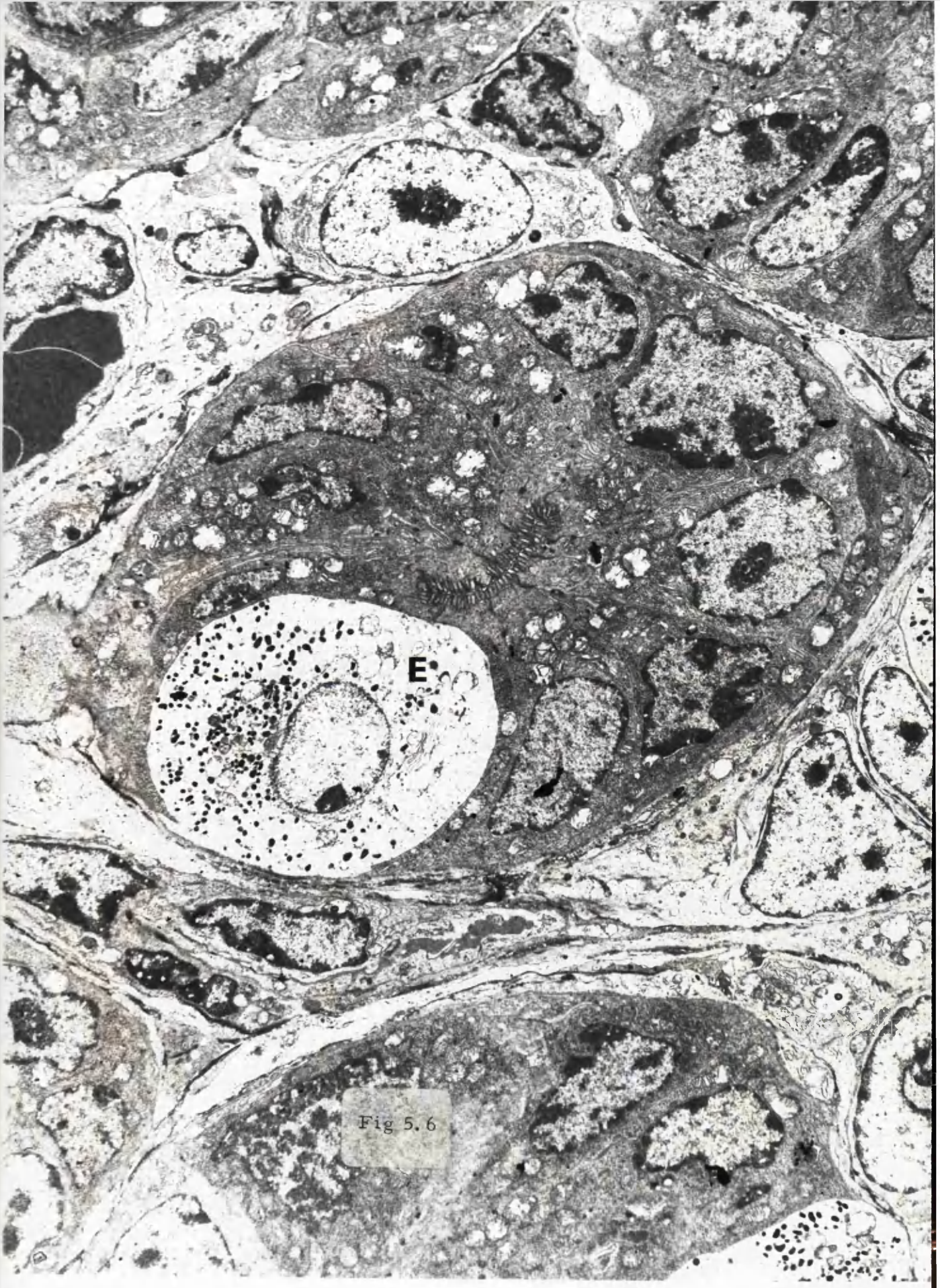

Fig 5.6

Fig 5.7

Fig. 5.8 Remnants of an isograft of foetal small intestine, implanted intraperitoneally within a millipore chamber 10 days previously.
H & E x 150

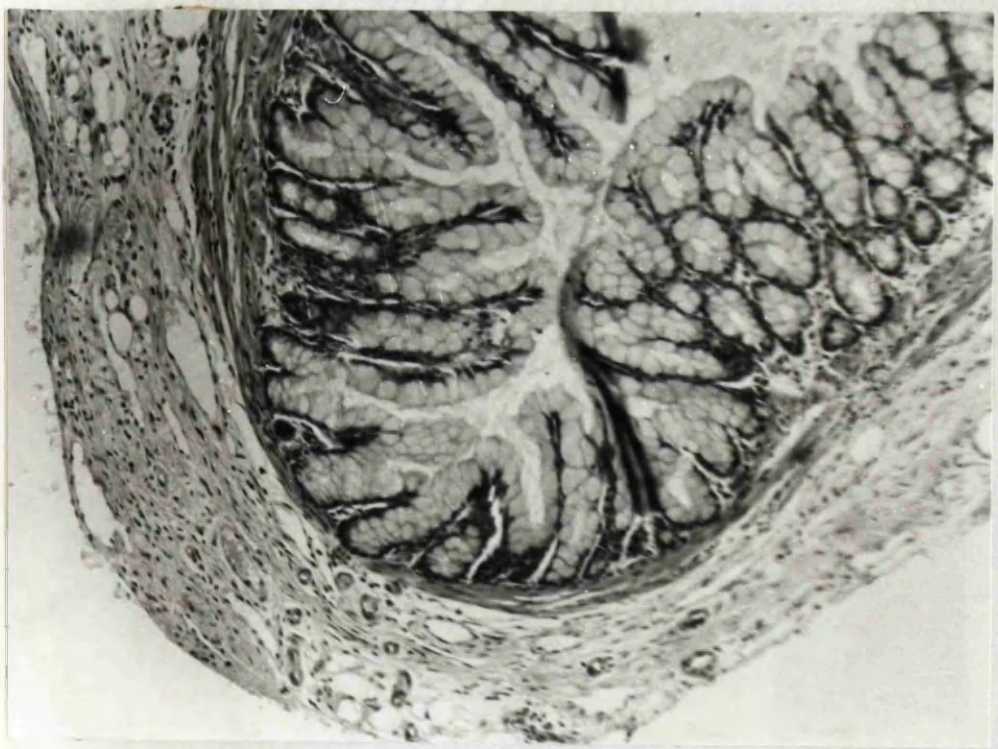

Fig. 5.9 Isograft of foetal colon in a CBA mouse, 31 days after implantation.
H & E x 150

Fig. 5.10 Isograft of small intestine in a Wistar rat, 26 days after implantation.
H & E x 370

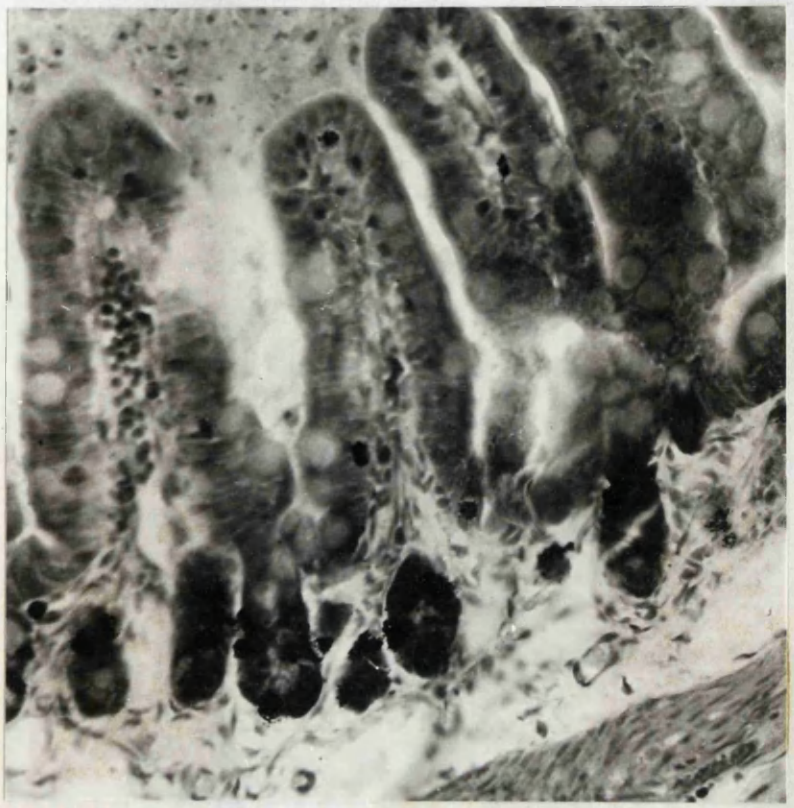

Fig. 5.11 Autoradiograph of an isograft of foetal small intestine in a CBA mouse, 38 days after implantation; 2 hours after an intraperitoneal injection of ^{3}H-thymidine. Labelled epithelial cells are present in the crypts and mucosal lymphocytes are also labelled.
MGP x 370

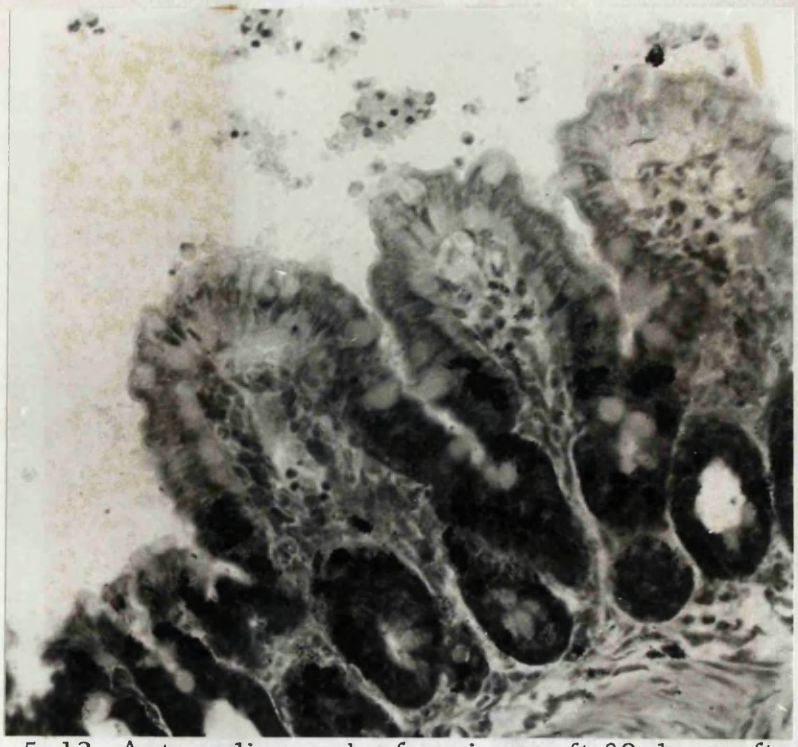

Fig. 5.12 Autoradiograph of an isograft 39 days after implantation, 24 hours after ^{3}H-thymidine. Labelled epithelial cells are present in the crypts and in the basal parts of the villi.
MGP x 370

Chapter 6

Influence of antigen deprivation on small intestinal lymphocytes

Normally the small intestinal lumen contains a vast array of antigens - foods, microorganisms, helminths and protozoal parasites. By contrast, isografts of foetal small intestine, although growing in an immunologically normal host, have never been exposed to these intraluminal antigens. In germ-free animals, the intestinal lumen contains only food antigens and dead microorganisms. Thus, comparison of lymphoid tissues of normal, germ-free and antigen-free intestine should allow the identification of the lymphocyte populations whose presence in the intestinal mucosa depends on intraluminal antigen exposure.

In the work described in this and subsequent chapters, the main emphasis has been on the size and morphology of Peyer's patches, and IF lymphocyte counts, in different experimental situations. The influence of antigen deprivation has been studied by morphological examination of the small intestine in 93 normal mice (CBA and BALB-c strains) aged 1 day to 6 months; 116 isografts of small intestine in CBA and BALB-c mice, from 1 to 86 days after implantation; and 10 sections of germ-free C3H mouse small intestine (loaned by Dr. J. C. Salomon). In all, a total of 154 grafts had been implanted, but in 38 cases satisfactory histological sections were not obtained, usually/

usually because of the very small size of graft submitted for processing.

In all the experimental groups, lymphocytes were found in the expected sites - Peyer's patches, lamina propria, and intraepithelially. However, there were clearcut differences between the numbers of lymphocytes found at these sites in young (pre-weaning) and older mice, and between normal intestine, germ-free and antigen-free intestines. Also, in some of the grafts of small intestine, there were collections of lymphocytes, apparently within lymphatic vessels in the loose connective tissue of the grafts and in endothelial-lined spaces under the kidney capsule adjacent to the grafts (Fig. 6.3). It is likely that these accumulations were due to obstruction of lymphatic drainage in the grafts.

Influence of antigen deprivation on Peyer's patches

The development of Peyer's patches in normally sited intestine. Tiny Peyer's patches could be detected in foetal intestine from the 18th day of gestation (Fig. 6.1). These contained mostly reticulum cells with a very few lymphocytes. By the 3rd day after birth Peyer's patches were well defined subepithelial accumulations of lymphocytes, and lymphocytes were present between the overlying epithelial cells. These Peyer's patches probably reflect the early emigration of T cells described by Joel et al (1971, 1972). Germinal centres started to appear at 4 weeks and were uniformly found in mice over the age of 5 weeks. Germinal centres appear to constitute at least three-quarters of Peyer's patch tissue in older animals.

The development of Peyer's patches in grafted intestine. Peyer's patches also appeared in the grafts implanted under the kidney capsule although they were often only 1/10-1/20 of the size of Peyer's patches in normally sited intestine. The earliest stage at which subepithelial aggregates were found was at 3 days after implantation, and in the 1st and 2nd weeksafter implantation there were many lymphocytes within the epithelium overlying the Peyer's patches. The small graft Peyer's patches showed a striking morphological difference when compared with Peyer's/

Peyer's patches in normal gut. Germinal centres, which are always present in normal gut, were never found in the graft Peyer's patches at any time after implantation. Indeed, it was the lack of germinal centres which accounted for most of the difference in size between graft and normal Peyer's patches. Figs. 6.2 and 6.3 illustrate typical graft Peyer's patches.

Peyer's patches in the intestine of germ-free mice. These were intermediate in size between the patches in normal and in antigen-free intestine. However, like the graft Peyer's patches, there were no germinal centres.

Influence of antigen deprivation on IE lymphocytes

The experimental schedule for IE lymphocyte counts is summarised in Table 6.1.

IE lymphocytes in normally sited intestine. In spite of the early appearance of aggregated lymphocytes in Peyer's patches, virtually no villous IE lymphocytes were found in either CBA or BALB-c mice until the 3rd week after birth (ie around the time of weaning). Thereafter the number of lymphocytes increased weekly and in the CBA mice plateauxed after the age of 6 weeks (Fig. 6.4). In both strains, the weekly increments were highly significant ($p < 0.01$) for the 3rd, 4th and 5th weeks after/

after birth, and significant ($p < 0.05$) in the 6th week (CBA mice).

IE lymphocytes in intestine of germ-free mice. The mean value for IE lymphocyte count in a group of 6 month old germ-free C3H mice was 3.3, with standard deviation (SD) of 1.4. Although these mice are from a different strain to the normal mice studied, the values are so much lower than in normal mice that it seems very likely that this is an exceptionally and significantly low value for a group of adult mice.

IE lymphocytes in grafted intestine. The grafts are vascularised within 1 or 2 days of implantation; however, lymphocytes did not appear in the epithelium until the grafted tissue had reached the age equivalent to 14-16 days after birth. Thereafter the graft lymphocyte counts increased each week, but by much smaller increments than normally sited intestine. When intraepithelial lymphocyte counts are plotted in relation to age (Figs. 6.5 and 6.6) it is clear that from the 3rd week the graft lymphocyte counts are lower than counts in conventionally sited intestine of the same age (CBA 3rd week - $P < 0.01$, 4th and subsequent weeks $P < 0.001$; BALB-c 3rd week $P < 0.003$; 5th week $P < 0.02$).

Lamina propria lymphocytes and plasma cells

I did not carry out formal counts of lamina propria lymphocytes and plasma cells. In normally sited and in grafted intestine they were/

were first detected in the 3rd week after birth or implantation. Their numbers increased with age, but in mature grafts and in germ-free gut there were relatively few lamina propria lymphoid cells, when compared with normally sited gut of the same age.

Conclusions

These experiments have shown that in the absence of intraluminal stimulation by antigens normally present in the gut there are profound effects upon all the lymphoid cells and tissues associated with the small intestinal epithelium of the mouse. When foetal gut was grafted under the kidney capsule of syngeneic adult recipients, lymphocytes appeared in all the expected sites - in Peyer's patches, in the lamina propria, between the epithelial cells of the villi and occasionally in transit across the villous epithelial basement membrane. However, their numbers were dramatically reduced when compared with normally sited gut of the same 'post-natal age', or with the host animal's own small intestine. Similar findings were obtained for the small group of germ-free mice, although, whereas germ-free and antigen-free IE lymphocyte counts were of the same order of magnitude, germ-free Peyer's patches were considerably larger than graft Peyer's patches. However, in neither of these groups were there Peyer's patch germinal centres.

Table 6.1 Experimental schedule for IF lymphocyte counts

Experimental Group	Nature of Tissue	"Post-natal age" of tissue (days)				Total
		-14	-28	-42	>42	
Normal CBA mice	small intestine (SI)	22	17	10	22	71
Normal BALB-c mice	SI	6	10	6	-	22
Axenic C3H mice	SI	-	-	-	10	10
Isografts in CBA mice	grafts of SI	11	29	30	13	83
Isografts in BALB-c mice	grafts of SI	13	11	9	-	33

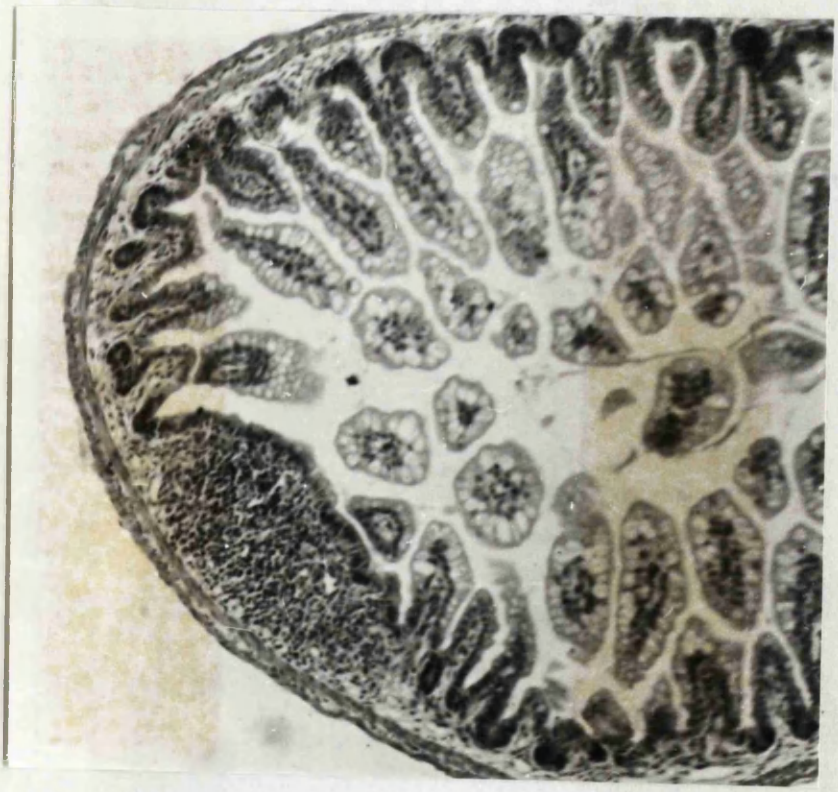

Fig. 6.1 Peyer's patch in the small intestine of a CBA mouse foetus at the 19th day of gestation.
H & E x 150

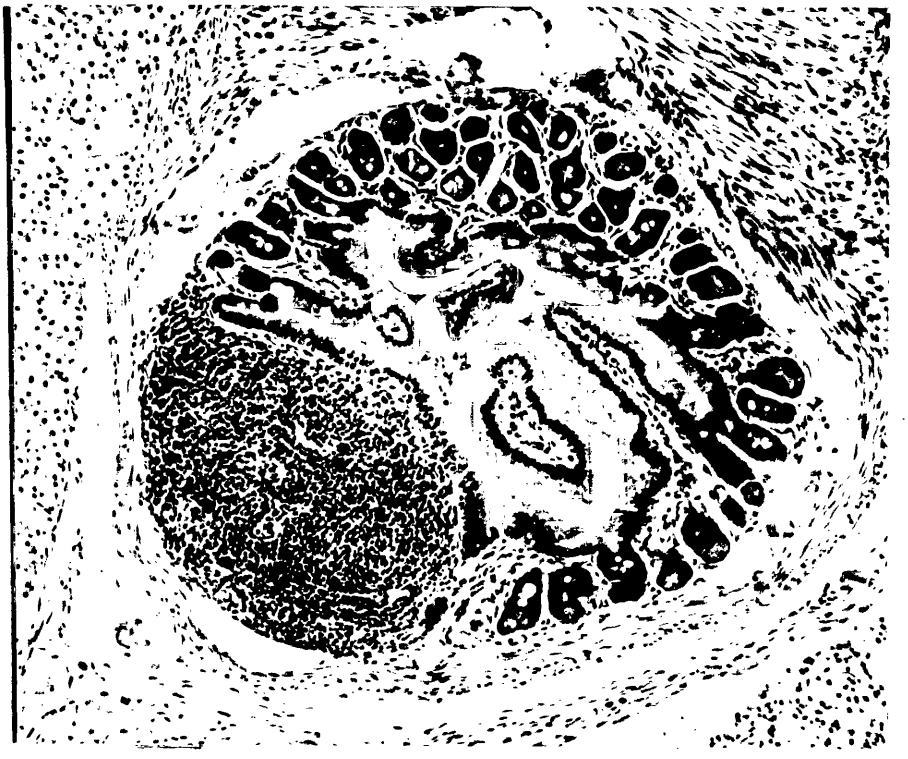

Fig. 6.2 Lymphoid nodule in an isograft of CBA foetal small intestine in a normal host; 33 days after implantation of the graft.
H & E x 150

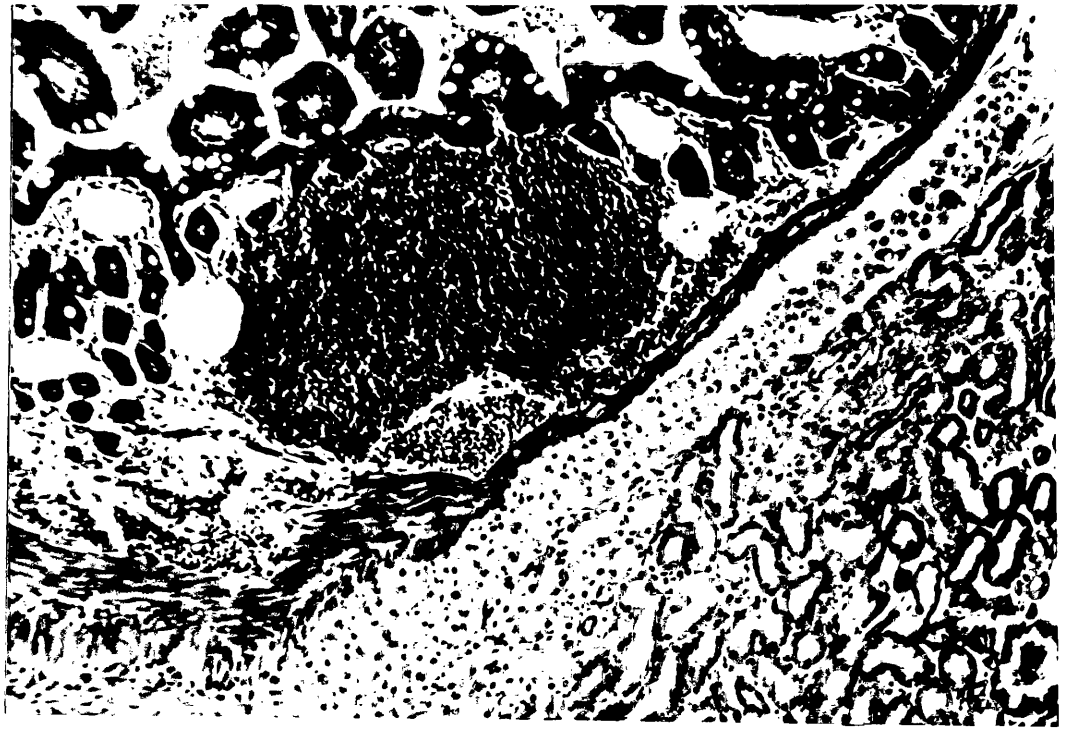

Fig. 6.3 Lymphoid nodule in an isograft in CBA host, 38 days after implantation.
H & E x 150

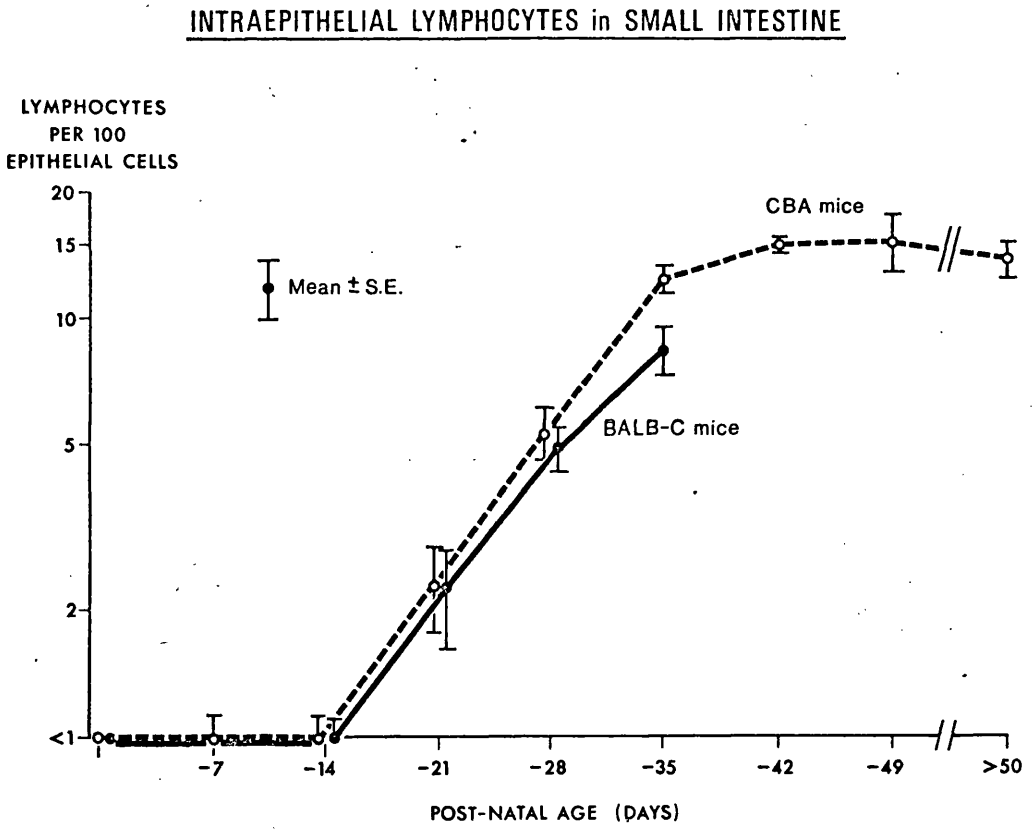

Fig. 6.4 IF lymphocyte counts related to tissue age in normally sited small intestine of CBA and BALB-c mice.

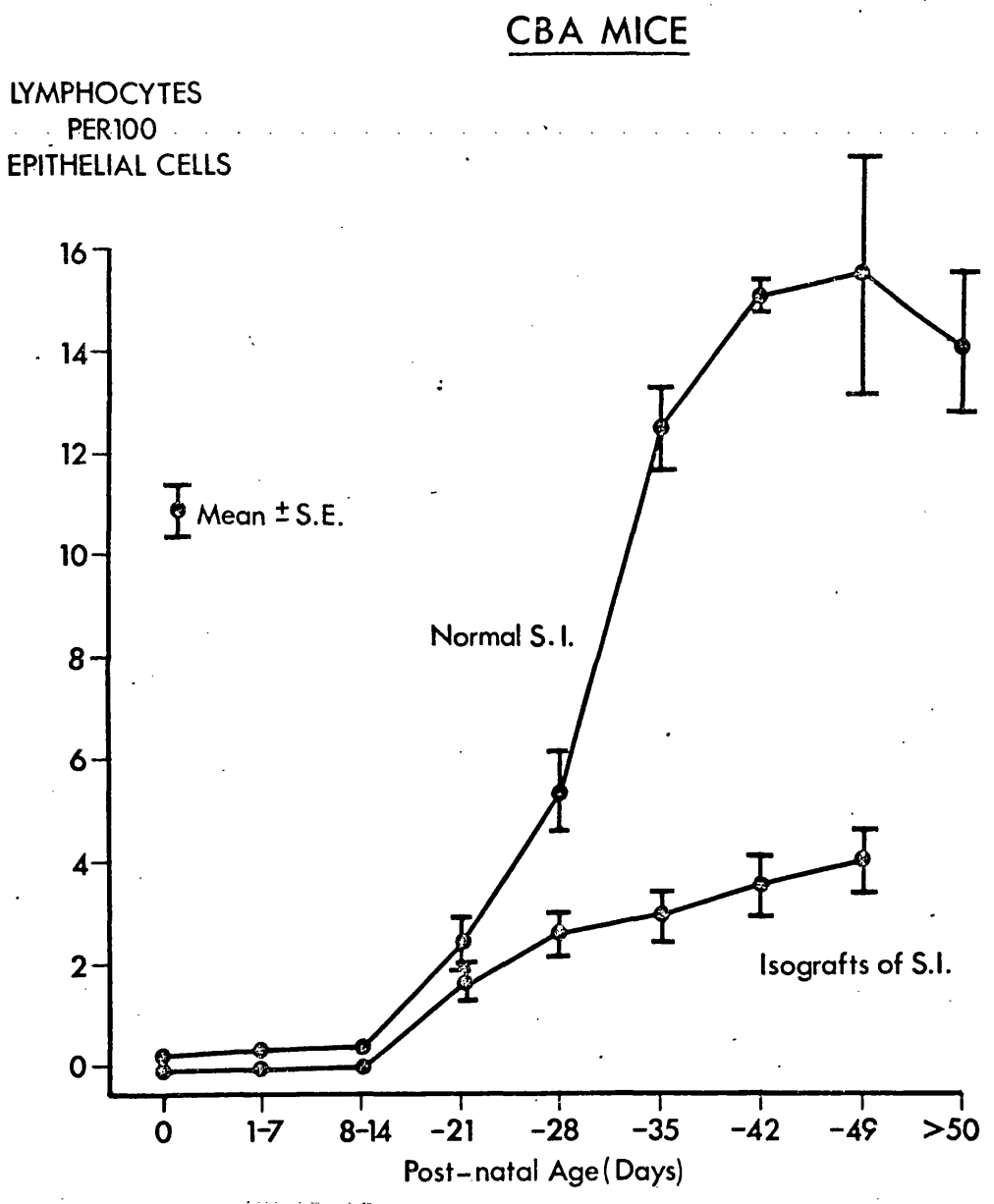

Fig. 6.5 IE lymphocyte counts related to tissue age in normally sited CBA mouse small intestine and in isografts of CBA foetal small intestine implanted in normal adult hosts.

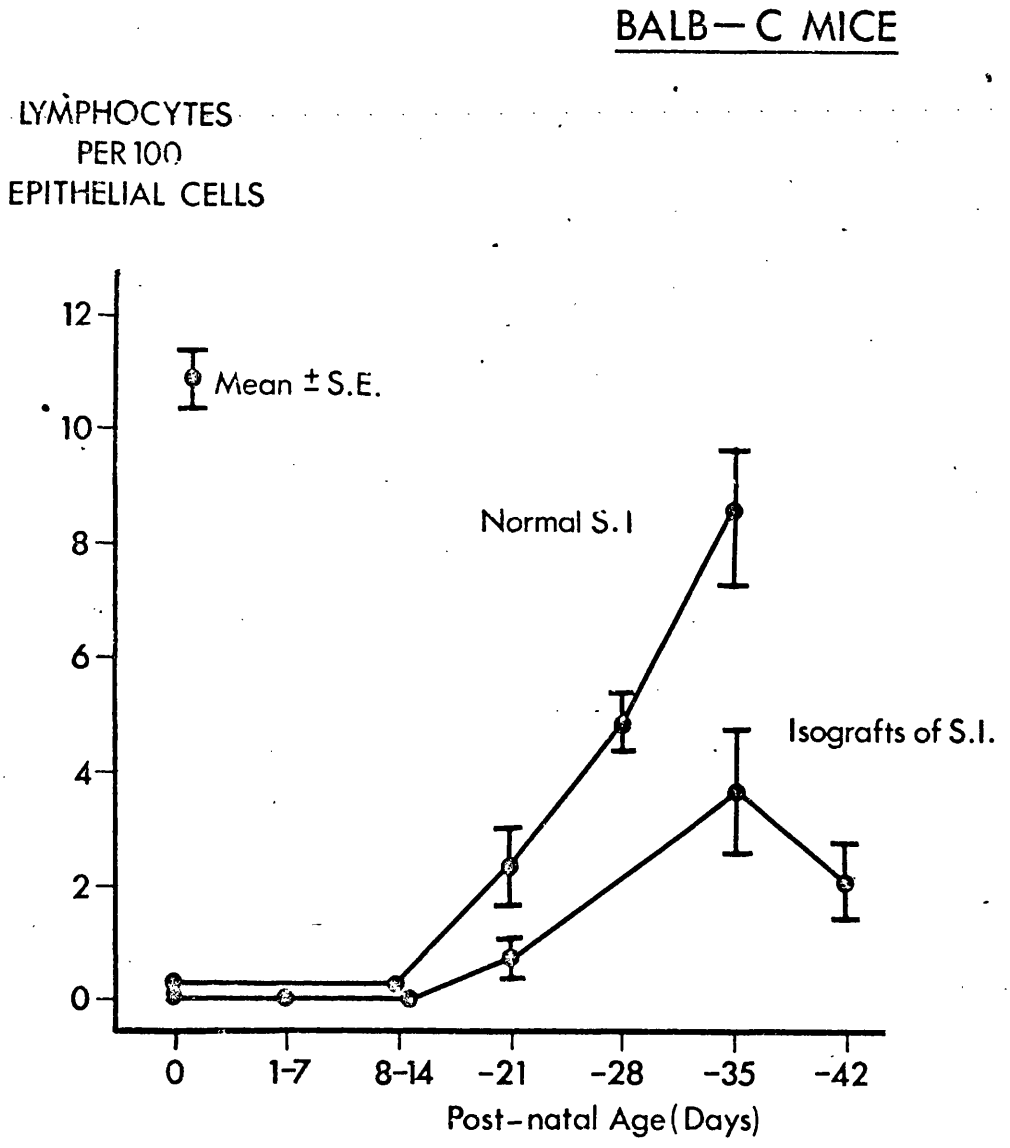

Fig. 6.6 IE lymphocyte counts related to tissue age in normally sited BALB-c mouse small intestine and in isografts of BALB-c foetal small intestine implanted in normal adult hosts.

Chapter 7

Thymus-dependent and thymus-independent lymphocytes in the small intestine.

Several workers have shown that Peyer's patches contain both thymus-dependent, T lymphocytes, and bone marrow derived, thymus-independent, B lymphocytes (Evans et al, 1967; de Sousa et al, 1969). In newborn mice almost all Peyer's patch lymphocytes are thymus derived (Joel et al, 1971, 1972; Chanana et al, 1973) but B lymphocytes predominate in older animals (de Sousa et al, 1969; Raff, 1971). I have attempted to demonstrate T and B intraepithelial lymphocyte populations by studying intestine from several types of "thymus-deprived" mice. The experimental schedule is summarised in Table 7.1.

Thymus-dependent and thymus-independent areas in Peyer's patches

There were small thymus-dependent areas (TDAs), depleted of lymphocytes, in the Peyer's patches of many of the thymus-deprived mice studied. Fig. 7.1 illustrates such an area in the Peyer's patch of a congenitally athymic mouse; Fig. 7.2 shows the relatively large TDA in the Peyer's patch of a germ-free, neonatally thymectomised mouse, and Fig. 7.3 shows a depleted TDA in an isograft of small intestine implanted in a thymus-deprived host. A post-capillary venule can be clearly seen in close relationship to the TDA. Such obviously depleted graft Peyer's patch TDAs were only occasionally seen, but in some of the grafts in thymus-deprived mice, the Peyer's patches were less densely packed with lymphocytes than was the case in normal animals.

Thymus-dependent and thymus-independent IF lymphocytes

Several groups of immunologically normal and thymus-deprived mice were compared.

Conventionally reared neonatally thymectomised mice. These specimens were loaned by Dr. D.M.V. Parrott. They were of small intestine from a group of C3H and C57xC3H mice which had/

had been thymectomised within 24 hours of birth, and from their normal healthy littermates. Results of IE lymphocyte counts are summarised in Fig. 7.4. It can be seen that in the neonatally thymectomised mice there were fewer IE lymphocytes than in their littermates, at the same age; these differences were significant for the age groups 36-42 days ($P < 0.001$) and 43-49 days ($P < 0.01$).

Conventionally reared thymectomised, irradiated, bone marrow reconstituted mice. A group of 19 adult thymus deprived mice were killed between 23 and 44 days after irradiation and reconstitution, and IE lymphocyte counts were compared with a group of 10 normal mice of the same age and reared at the same time. Results are summarised in Table 7.2. Values were significantly lower in the thymus-deprived mice ($P < 0.001$). However, this finding could be interpreted either as the absence of a T population in these mice, or simply as a result of slow recovery from irradiation.

Germ-free neonatally thymectomised mice. (Specimens loaned by Dr. J.C. Salomon). Sections of intestine were available from 10 untreated germ-free C3H mice, and 25 neonatally thymectomised germ-free mice. Results of IE lymphocyte counts are given in Table 7.3. It can be seen that, even though there are few IE lymphocytes/

lymphocytes in the untreated germ-free mice, the values were even lower (significantly so, $P < 0.00001$) in the neonatally thymectomised germ-free mice.

Grafts of small intestine in thymus-deprived hosts. IE lymphocytes appeared in grafts in thymus-deprived mice in the 3rd week after implantation, and, although their numbers were lower than for grafts in immunologically intact hosts (Fig. 7.5) these differences are not significant.

Conclusions

These experiments have confirmed that there are small thymus-dependent areas, depleted of lymphocytes, in Peyer's patches of conventional, germ-free and antigen-free intestine. The TDAs in germ-free mouse Peyer's patches were very prominent and formed up to half of the area of the patch in histological sections.

Also, the consistent findings of significant differences between IE lymphocyte counts in intact and thymus-deprived mouse intestine confirms and extends a previous report (Fichtelius et al, 1968) and demonstrates that there are both T and B intra-epithelial as well as Peyer's patch lymphocytes. Also, T lymphocytes form a relatively greater proportion of the total in young animals, and in germ-free mice.

Table 7.1 Experimental schedule for detection of thymus-dependent and thymus-independent lymphocyte populations.

Experimental Group	Nature of tissue	Number of specimens examined: Immunologically intact	Number of specimens examined: Thymus-deprived
Normal CBA mice	Small intestine (SI)	10	
Thymectomised, irradiated, bone marrow reconstituted CBA mice	SI		19
Normal littermates of neonatally thymectomised mice	SI	17	
Neonatally thymectomised C3H and C57x C3H mice	SI		23
Littermates of congenitally athymic mouse	Peyer's patch	2	
Congenitally athymic mouse	Peyer's patch		1
Isografts in CBA mice	Grafts of SI	83	
Isografts in thymus-deprived CBA mice	Grafts of SI		32
Germ-free C3H mice	SI	10	
Neonatally thymectomised germ-free C3H mice	SI		20

Table 7.2 IE lymphocyte counts in normally sited SI of CBA mice

Immunological Status	Number of mice	age (days)	IE lymphocytes per 100 epithelial cells		
			mean	SD	
normal	10	70-120	28.86	7.30	
thymectomised, irradiated, bone marrow reconstituted	19	70.110	18.05	6.23	$P < 0.001$

Table 7.3 IE lymphocyte counts in normally sited SI of germ-free C3H mice aged 6 months.

Immunological Status	Number of mice	IE lymphocytes per 100 epithelial cells		
		mean	SD	
normal	10	3.32	1.43	$P < 0.00001$
neonatally thymectomised	25	1.47	0.57	

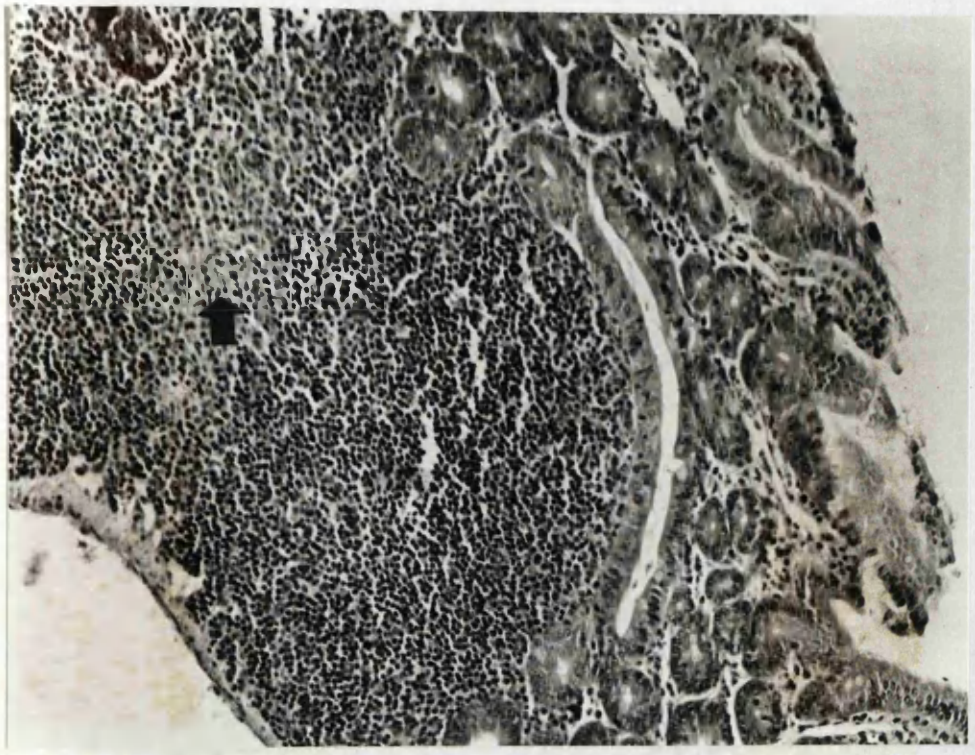

Fig. 7.1 Part of a Peyer's patch from a congenitally athymic mouse. TDA, arrowed, is depleted of lymphocytes. H & E x 150

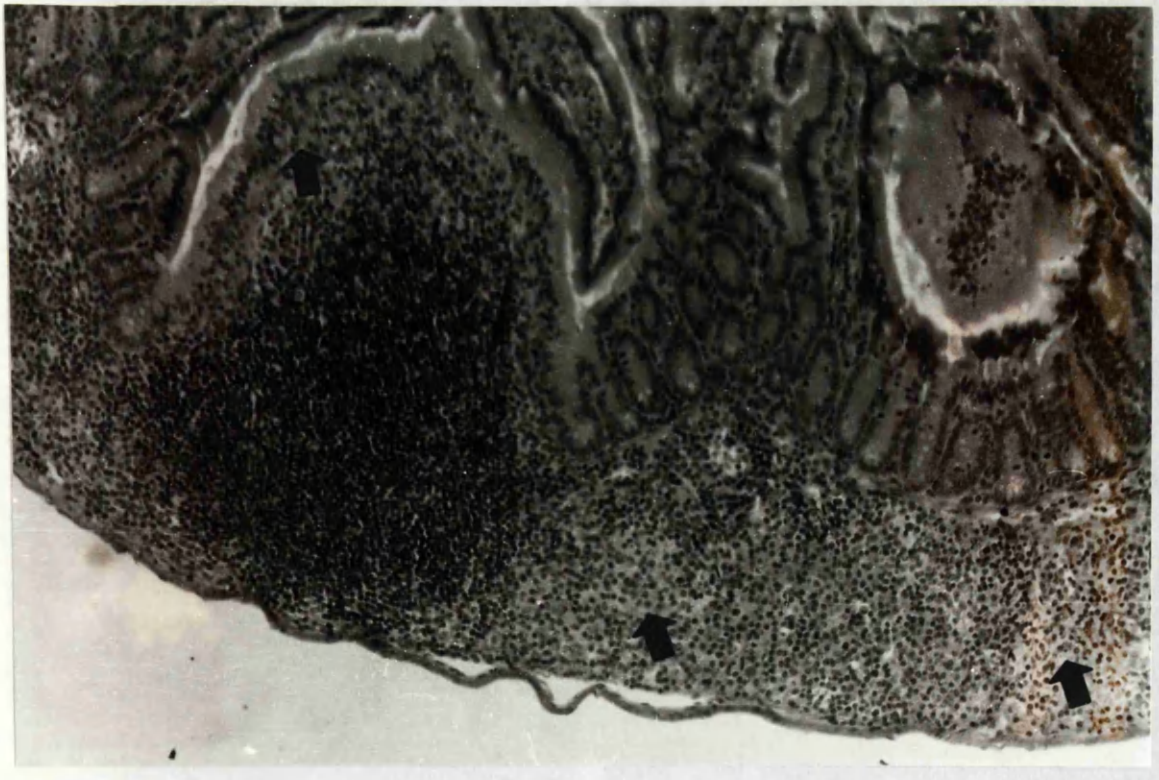

Fig. 7.2 Part of a Peyer's patch from a germ-free neonatally thymectomised C3H mouse. The extensive TDA, arrowed, is depleted of lymphocytes. H & E x 150

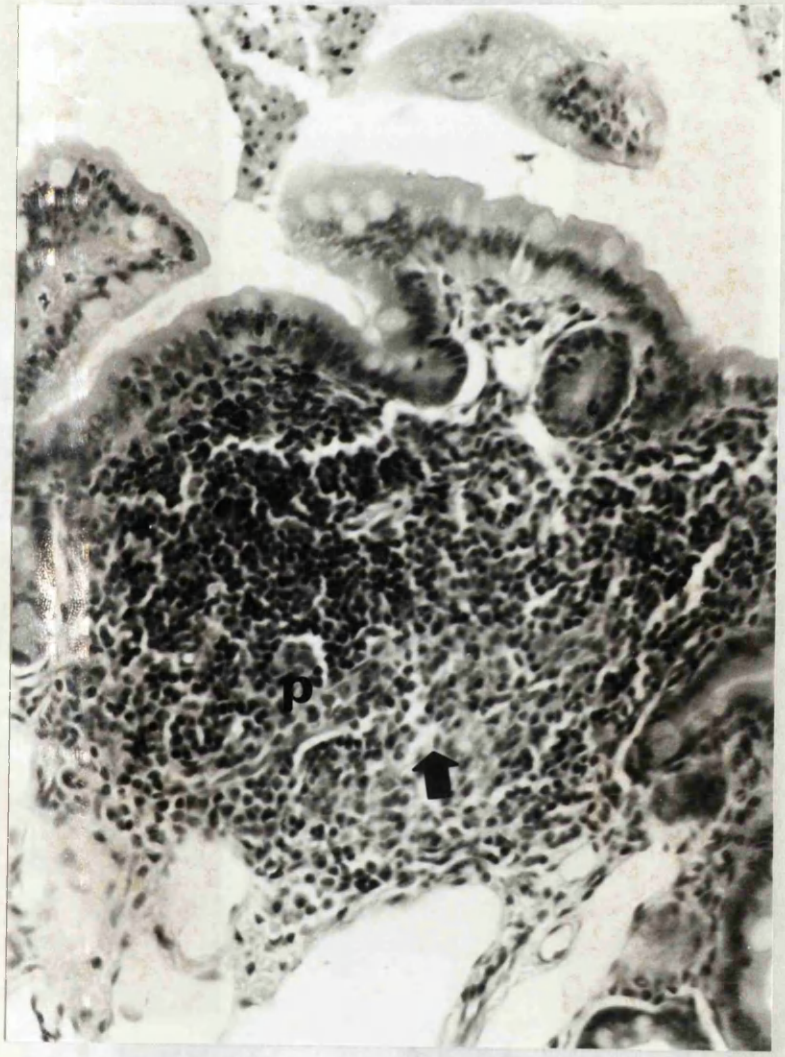

Fig. 7.3 Peyer's patch in an isograft of CBA small intestine, 41 days after implantation into a thymus-deprived CBA mouse. The depleted TDA is arrowed. P - post capillary venule.
H & E x 370

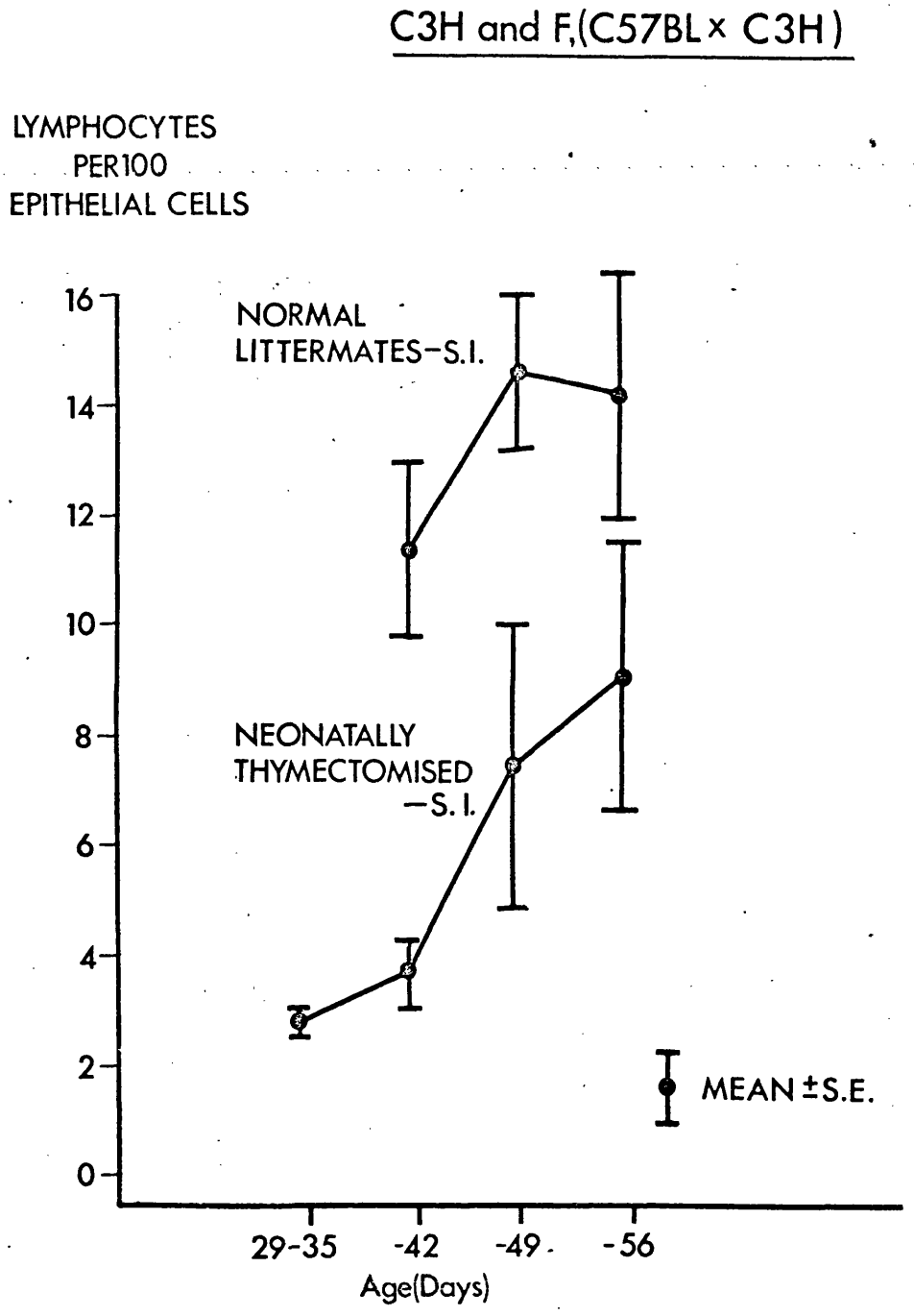

Fig. 7.4 IE lymphocyte counts related to age in the small intestine of neonatally thymectomised mice, and of untreated littermates.

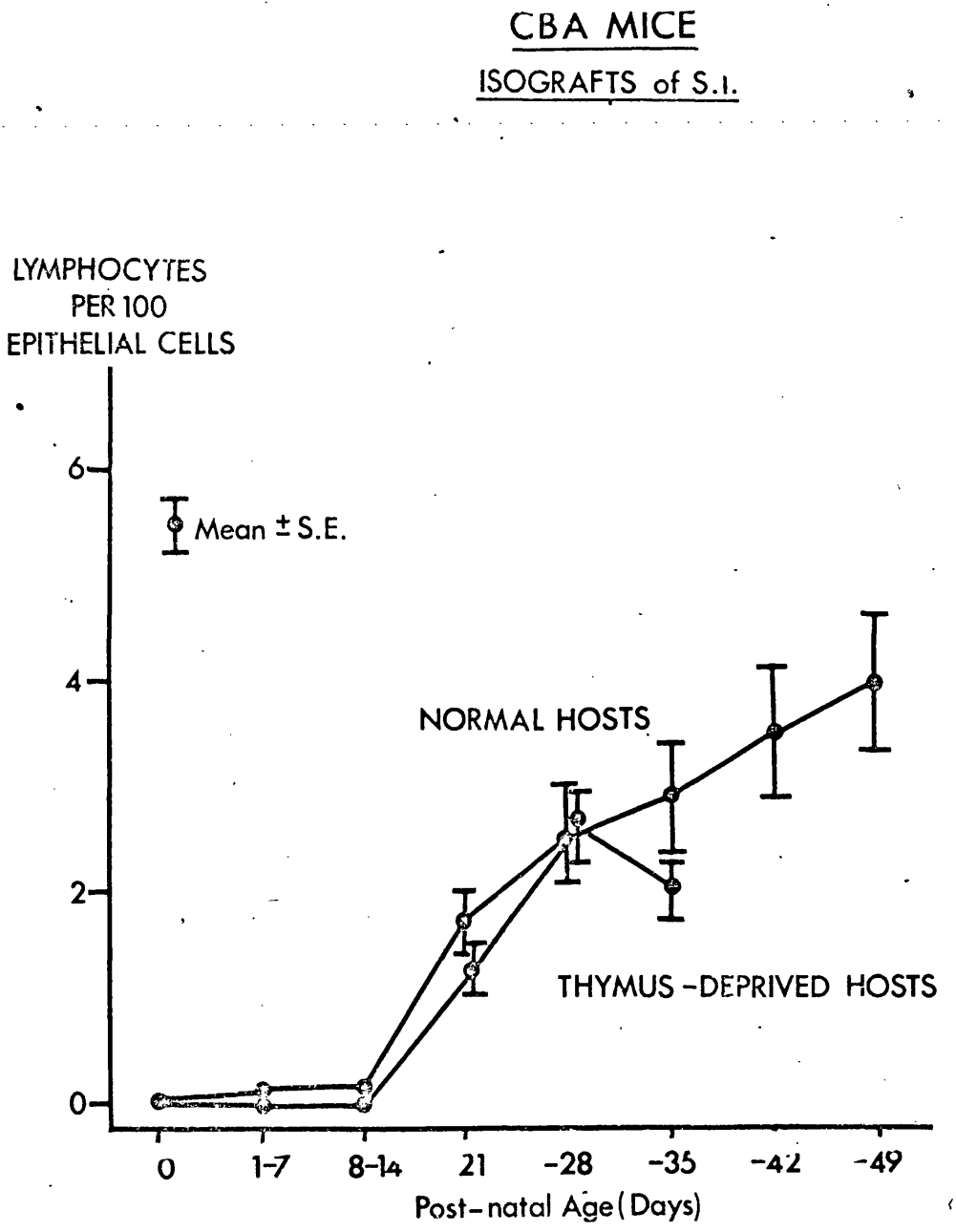

Fig. 7.5 IE lymphocyte counts related to tissue age in isografts of CBA foetal small intestine implanted in thymus-deprived and immunologically normal hosts.

Chapter 8

Migration of lymphocytes within the small intestine.

Isografts of foetal small intestine are usually devoid of lymphoid tissue when implanted; however, lymphocytes appear in Peyer's patches and mucosa shortly after implantation in adult hosts, and it is likely that these have migrated from the blood stream into the graft. It is already known that isotopically labelled small lymphocytes will enter Peyer's patches with the same facility as they enter lymph nodes and spleen (Gowans and Knight, 1964; Goldschneider and McGregor, 1968a, 1968b; Griscelli et al, 1969; Parrott and de Sousa, 1971). Cells which migrate to the normal lamina propria are a completely distinct group; they are the large lymphocytes of lymph, mesenteric lymph nodes or thymus, and can be labelled with ^{3}H-thymidine. (Gowans and Knight, 1964; Goldschneider and McGregor, 1968a, 1968b; Delorme et al, 1969; Griscelli et al, 1969; Hall and Smith, 1970).

This chapter describes a series of experiments in mice which I carried out with Professor D. M. V. Parrott; they were designed to examine the migration of T and B lymphocytes, and immunoblasts, to small intestine (either normally-sited or antigen-free).

Design of cell migration experiments

The experimental schedule is summarised in Table 8.1.

Cell suspensions were prepared and labelled with ^{3}H adenosine or ^{3}H thymidine, *in vivo* or *in vitro*, as described in Chapter 3. Between 95% and 100% of cells were labelled by incubation with ^{3}H adenosine, and the vast majority were small or medium lymphocytes. In the *in vitro* ^{3}H thymidine labelled cell preparations only medium and large lymphocytes were labelled, most having 30-50 grains per cell. In the *in vivo* ^{3}H thymidine labelled preparations there were similar heavily labelled medium and large lymphocytes but also some lightly labelled small lymphocytes ($<$ 10 grains per cell) which were disregarded in the estimates of injected labelled cells. The proportion of ^{3}H thymidine labelled cells varied from less than 1% in unstimulated lymph node cells, to 10-15% in thymus and stimulated lymph node preparations.

Recipient mice were normal (N) or thymus deprived (B) CBA mice, each with two well established small intestinal isografts implanted 5-12 weeks previously. (There was one group of recipients without grafts; see Table 8.1). Labelled cells ($1 - 9 \times 10^{7}$ per recipient) were injected intravenously and the mice/

mice killed 24-72 hours later. Autoradiographs were prepared from sections of thymus, spleen, mesenteric and inguinal lymph nodes, Peyer's patches, three segments of small intestine and two subcapsular grafts of small intestine.

Identification and quantitation of labelled cells. Most of these counts were done by Professor D. M. V. Parrott. In Peyer's patches and lymph nodes, the number of labelled cells per unit area was determined by using a graticule eyepiece (see Chapter 3). Several such unit areas were examined in each section, both in thymus-dependent and thymus-independent areas. The division of Peyer's patches into compartments was based on the observations described in Chapter 7. TDAs included the interfollicular areas and subepithelial zones; thymus-independent areas comprised the primary nodules and cuffs of lymphocytes surrounding germinal centres (nodular areas, NAs). Cells within germinal centres were noted but not included in the net counts. Graft Peyer's patch area counts were not divided into thymus-dependent and nodular areas. Finally, in each gut preparation, about 40 villi were scanned for labelled cells.

Distribution of spleen and thymus cells in Peyer's patches

Spleen cells from normal and B donors were labelled in vitro with ^{3}H adenosine and injected into normal and B recipients. ($2x10^{7}$ cells/

($2x10^7$ cells per mouse). Table 8.2 summarises the counts of labelled cells (per unit area) found in sections of graft and normal Peyer's patches. Analysis of the results for normal Peyer's patches showed that the numbers and distribution patterns of labelled cells were influenced by several factors including donor cell type, and the immunological status of the host. Normal spleen cells, with around 35% T and 65% B (Parrott and de Sousa, 1971) were found both in nodular and thymus-dependent areas of the Peyer's patches. When the inoculum consisted of spleen cells from B mice (95% B) there was a significant reduction in the number of cells in the TDAs ($P < 0.001$) but not in the nodular areas. In addition it seems that the recipient can influence the number of cells per unit area, for more cells were found in all areas of Peyer's patches in thymus deprived recipients when compared with normal recipients.

Labelled spleen cells were found in all the graft Peyer's patches and, from the unit area counts (Table 8.2) it is evident that both normal and B spleen cells gained access to graft Peyer's patches as readily as to normal gut Peyer's patches. In some cases, labelled lymphocytes were identified in lymphatic vessels adjacent to graft Peyer's patches.

Thymus cell traffic to the TDAs of Peyer's patches has been reported previously (Parrott and de Sousa, 1969). These autoradiographs were re-examined and recounted for the purposes of comparison with the spleen cell experiments described above. The results are summarised in Table 8.3 and show that ^{3}H adenosine labelled thymus cells were almost entirely confined to the TDAs of Peyer's patches. Very few large thymocytes, labelled with ^{3}H thymidine, found their way to Peyer's patch tissue and the numbers were too low for meaningful unit area counts.

Thus, both in patterns of distribution and in numbers of cells per unit area these results for the homing of normal spleen cells, B spleen cells and thymus cells within Peyer's patches are directly comparable to results obtained in other lymphoid tissues - spleen, inguinal and mesenteric lymph nodes (Parrott and de Sousa, 1971; Parrott et al, 1973).

Distribution of spleen and thymus cells in the lamina propria

Small numbers of normal and B spleen and thymus cells were found in the lamina propria of villi very close to Peyer's patches but virtually none were found further away from Peyer's patches and there were no labelled cells between the epithelial cells of the villi. There were no ^{3}H thymidine labelled large thymocytes in the lamina propria.

Distribution of ^{3}H-thymidine labelled lymph node cells

"Primed" lymph node cells, labelled with ^{3}H thymidine, were obtained in three ways.

(1) Cells primed against the contact sensitising agent, oxazolone, were obtained from auricular lymph nodes 3 days after oxazolone had been painted on the ears of the donor mice. Control suspensions of lymphocytes were prepared from other lymph nodes distant to the site of sensitisation, including inguinal and mesenteric.

(2) Cells primed against the gut parasite Nippostrongylus brasiliensis were obtained from mesenteric lymph nodes of mice 6 days after subcutaneous injection of larvae. Mesenteric lymph node cell suspensions from uninfected donors of the same age were also prepared and labelled.

(3) cells primed against food antigens were obtained from the mesenteric lymph nodes of nestling mice, ie in the third week of life, at the time when the young mice first sample solid food and so are likely to be in the early stages of an immune response to dietary antigens.

Only the suspensions of oxazolone-primed cells, Nippostrongylus-primed cells and cells from nestling mice contained sufficient numbers of labelled cells to be detected within recipients.

Labelled/

Labelled cells were found in Peyer's patch tissue in the normal as well as grafted gut but there was no clear evidence of preference for different areas (Table 8.4). The number of labelled cells per unit area was lower than with spleen cell inocula, but fewer labelled cells had been injected (Table 8.1). It is interesting to note (Table 8.4) that the auricular node cells found lymph nodes, particularly inguinal lymph nodes, more attractive than did the mesenteric node cells.

These differences in migrating behaviour between the oxazolone primed auricular and mesenteric lymph node cells were accentuated by the results of scanning the lamina propria for labelled cells. Labelled mesenteric lymph node cells from young nestling donors and from Nippostrongylus infected donors were regularly found in the villi of both the normal and grafted gut (Fig. 8.1) with about the same frequency. The cells, some of which had the form of plasma cells, were all in the lamina propria and none have so far been found between the epithelial cells of the villi. Virtually no labelled cells from the auricular lymph nodes were found in the lamina propria of the villi of normal or grafted gut. Only on rare occasions were one or two labelled small lymphocytes found in villi close to Peyer's patches.

Conclusions

These experiments have confirmed, in mice, that different categories of lymphocytes migrate to the Peyer's patches and to the mucosa. Small lymphocytes, from normal spleen, B cell enriched spleen or thymus, migrate to Peyer's patches of normal or antigen-free gut. ^{3}H thymidine labelled lymphoblasts from primed mesenteric lymph node (but not from primed peripheral lymph node) migrate to the lamina propria of the villi. As yet no labelled cells have been traced to an intraepithelial site.

Table 8.1 Experimental schedule for studies of lymphocyte migration to the small intestine

Donor Cells	Isotope Label	Dose of Cells	Number of labelled cells injected	Recipients (with grafts) N - normal B - thymus-deprived	
Normal spleen	in vitro ^{3}H adenosine	2×10^{7}	2×10^{7}	3N	3B
B spleen	in vitro ^{3}H adenosine	2×10^{7}	2×10^{7}	3N	3B
Normal adult thymus	in vitro ^{3}H adenosine	4.5 or 9×10^{7}	4.5 or 9×10^{7}	4N*	4B*
Nestling thymus	in vivo ^{3}H thymidine	4×10^{7}	2.6×10^{7}	2N	2B
Normal adult thymus	in vitro ^{3}H thymidine	6.5×10^{7}	1×10^{7}	3N	
Unstimulated inguinal node	in vitro ^{3}H thymidine	3×10^{7}	0.9×10^{5}	3N	
Stimulated auricular node (oxazolone)	in vitro ^{3}H thymidine	3×10^{7}	1.9×10^{6}	4N	
Unstimulated mesenteric node, nestling	in vivo ^{3}H thymidine	1.7×10^{7}	2.5×10^{6}	2N	2B
Unstimulated mesenteric node, normal adult	in vitro ^{3}H thymidine	1.7×10^{7}	1.7×10^{5}	4N	
Stimulated mesenteric node (Nippostrongylus infested adult)	in vitro ^{3}H thymidine	3×10^{7}	0.9×10^{6}	6N	

* This group of recipients did not have antigen-free gut grafts.

Table 8.2 Distribution of ^{3}H adenosine labelled spleen cells from normal (N) and thymus-deprived (B) donors, in Peyer's patch tissue

Donor	Recipient	Number of labelled cells per unit area (4 weeks exposure)								
		Normal Gut Peyer's patch						Graft Peyer's patch		
		NA			TDA					
		n	mean	SE	n	mean	SE	n	mean	SE
N	N	10	5.7	0.8	7	5.3	0.8	9	5.2	1.1
N	B	5	8.0	0.3	3	23.3	1.8	15	6.7	1.0
B	N	13	4.8	0.5	7	1.7	0.3	6	0.7	0.3
B	B	5	8.6	1.0	18	4.9	1.0	7	6.6	1.0

Three recipients per group were killed 48 hours after i.v. injection of 2×10^{7} spleen cells from N or B donor.

n - number of unit areas counted.

Table 8.3 Distribution of ^{3}H adenosine labelled thymus cells in normally sited Peyer's patches

Number of labelled thymus cells injected	Recipient	Number of labelled cells per unit area (10 week exposure)					
		Nodular area			Thymus dependent area		
		n	mean	SE	n	mean	SE
4.5×10^7	N (killed 48 hours later)	10	0		9	2.7	0.4
4.5×10^7	B (killed 48 hours later)	19	0.1	0.7	18	3.6	0.4
9.0×10^7	N (killed 72 hours later)	5	0		5	1.8	0.5
9.0×10^7	B (killed 72 hours later)	10	0.2	0.1	10	5.8	1.0

Table 8.4 Distribution of ^{3}H thymidine labelled, sensitised lymph node cells, in lymphoid tissues

Treatment of donor	Number of labelled cells injected	Number of labelled cells per unit area (means; 9 week exposure)						
		Inguinal LN		Mesenteric LN		Normally sited Peyer's patch		Gut Graft Peyer's patch
		NA	TDA	NA	TDA	NA	TDA	
Oxazolone sensitisation	1.9×10^{6} auricular LN cells	0.3	2.2	0.1	1.2	0.02	0.4	0.5
Nippostrongylus infection	0.94×10^{6} mesenteric LN cells	0.1	0.2	0.1	0.4	0.02	0.4	0.7

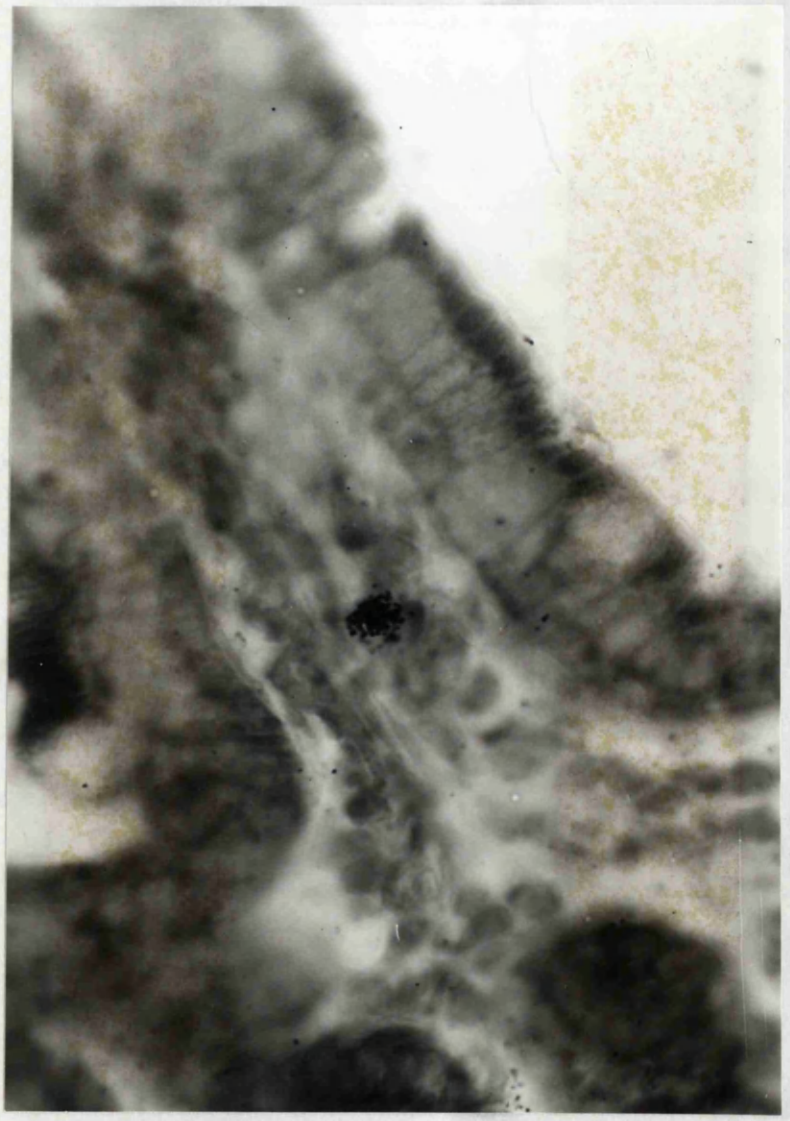

Fig. 8.1 Labelled cell in lamina propria of the normally sited small intestine. The mouse had received ^{3}H thymidine labelled mesenteric lymph node cells from N brasiliensis primed donors, 24 hours previously.
MGP x 1000

Chapter 9

Rejection of allografts of small intestine

The technique of heterotopic implantation of foetal small intestine has proved suitable for experimental studies of rejection of allografts, as well as for the previously reported studies on isografts. Since graft lumina contain neither food nor commensal microorganisms, pathological changes observed can be attributed entirely to the rejection process. Also, since there is a paucity of lymphocytes in the small intestine of the neonatal mouse, the abnormal lymphocyte infiltration of early rejection should be easily recognised.

The influence of histocompatibility differences on the rejection process has been examined by grafting gut between strains which are H2 identical (CBA and C3H-Bi) and between strains which differ at the H2 histocompatibility locus (CBA and BALB-c) (Snell et al, 1964). Also, since it is known that survival of skin grafts is prolonged in thymus-deprived mice (Miller et al, 1963; Cross et al, 1964) the survival of a group of BALB-c grafts implanted in thymus deprived CBA recipients has been studied.

Table 9.1 summarises the numbers of allografts studied. Of 214 grafts implanted, 210 were found macroscopically at post-mortem examination and satisfactory histological preparations were obtained for 173 grafts. The remaining 37 grafts, which were randomly distributed throughout all the groups, could not be assessed satisfactorily because insufficient graft tissue remained for sectioning.

Histopathology of rejection

There was considerable variation in the morphology of grafts, even in groups of tissues studied at the same time after implantation. Nevertheless a consistent, predictable pattern of rejection emerged. The same events occurred in sequence in all experimental groups although the time occupied by the different phases varied according to the strain combination. Grafts developed normally for a few days after implantation; they had well defined short crypts, normal villi and regular columnar epithelium (grade: "normal"). At this age the lamina propria of normal mouse intestine contains no plasma cells and virtually no lymphocytes (Chapter 6).

The earliest sign of graft rejection was the appearance of lymphocytes in the loose connective tissue of the lamina propria, with accumulation of lymphocytes in the lymphatic vessels. Pyroninophilic blast cells could be seen in some of the larger lymphatics (grade: "L+") (Fig. 9.1). Later, there was lymphocyte infiltration of the full thickness of the grafts, including the epithelium and the muscle layers of the submucosa. The heaviest lymphocyte infiltration persisted in the lamina propria. Some grafts contained large, dense aggregates of lymphocytes which could have entered the grafts at the site of the tiny Peyer's patches, found in isografts of the same age, and which

do/

do contain post-capillary venules. In the allografts, the lymphocyte aggregates contained many blast cells, but no germinal centres were seen.

The general pattern of crypts and villi varied considerably from graft to graft and even within a single graft. Some tissues had virtually normal crypts and villi with normal columnar epithelium; in others, the graft was lined with an undulating monolayer of cuboidal or columnar epithelial cells. Grafts with these appearances were graded "L++". However, many tissues had a strikingly abnormal mucosal pattern with long crypts and low or absent villi, ie partial or subtotal villous atrophy. These were classified as grade "Flat" (Fig. 9.2). Other interesting features were seen at this stage of rejection. Many crypts contained amorphous debris; in a few grafts there were apparent breaks in the continuity of the basal lamina and epithelial cell layer, with extrusion of small groups of cells into the lumen of the graft. In several grafts, the lumen contained sheets of epithelial cells resembling "casts" of villous epithelium (Fig. 9.3).

In late rejection (Grade "Submucosa") the epithelium had been destroyed and the grafts consisted of smooth muscle heavily infiltrated with lymphocytes and plasma cells (Fig. 9.4). Serial sections/

sections of these grafts sometimes revealed small scraps of cuboidal epithelium with a few shallow crypts.

Time-course of rejection

After the pathological stages of rejection had been defined, the sections were coded, mixed and reviewed, and each was categorised into one of the five pathological grades described above: normal, L+, L++, flat, submucosa. The progression of the rejection process in the four strain combinations studied: CBA → C3H; C3H → CBA; CBA → BALB-c; BALB-c → CBA is summarised in Figs. 9.5 - 9.8. It can be seen that grafts between CBA and BALB-c (H-2 incompatible) were rapidly rejected - no normal grafts were found after 3 days, and all specimens showed complete rejection by 12 days. In contrast, with grafts between CBA and C3H (H-2 compatible) the first signs of rejection were seen at 5 days and some tissues were still not completely rejected at 3 weeks after implantation.

Rejection of allografts in thymus-deprived hosts

The rejection process in grafts of BALB-c foetal intestine implanted in thymus-deprived CBA mice was often significantly delayed (Fig. 9.9). Even though donor and host strains were H-2/

H-2 incompatible, half of the grafts showed no signs of rejection when examined up to 71 days after implantation. These grafts retained normal morphology, at times when there would be complete rejection of BALB-c grafts in immunologically normal CBA mice (Fig. 9.10). These tissues have been populated by presumably thymus-independent lymphocytes and plasma cells, and yet are very similar in appearance to syngeneic grafts of the same age.

Conclusions

This series of experiments has shown that allografts of intestine, implanted under the kidney capsule, will grow for a few days but thereafter a predictable histological pattern of rejection ensues.

There is, initially, lymphocyte infiltration of the lamina propria. Later, lymphocytes and blast cells infiltrate the full thickness of the intestine and there may be reduction of the villous-crypt ratio, giving the mucosa a coeliac-like appearance. Finally, the epithelium is destroyed, leaving only sheets of smooth muscle heavily infiltrated with lymphocytes and plasma cells. These same events occurred in sequence in all the strain combinations studied but the time course of rejection varied with histocompatibility differences between donor and host strains, /

strains, and thymus-deprived mice showed a profoundly impaired ability to reject allografts.

Table 9.1 Details of donor-host strain combinations used to determine the histopathology of rejection of small intestinal allografts.

Donor strain : H2	Host strain: H2	Number of allografts implanted	Number suitable for histological assessment
CBA k	C3H k	48	38
C3H k	CBA k	34	28
CBA k	BALB-c d	54	45
BALB-c d	CBA k	54	41
BALB-c d	Thymus-deprived CBA k	24	21

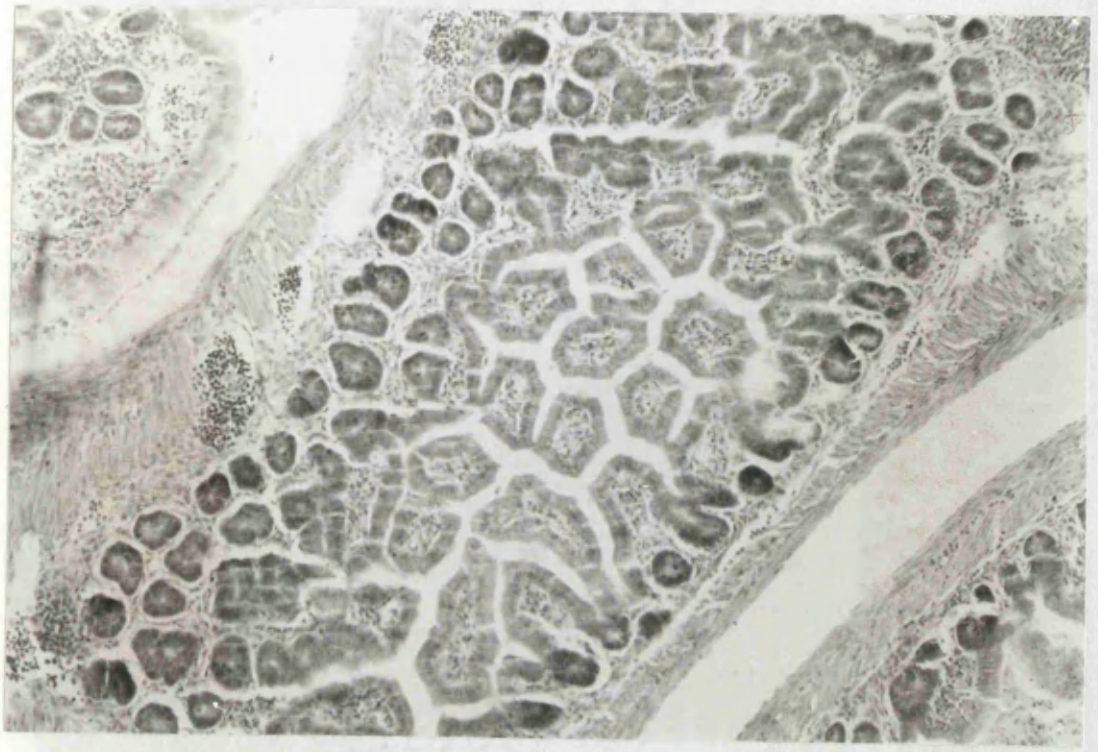

Fig. 9.1 Early rejection, grade L+. Lymphocytes are present in the lamina propria and within the lymphatic vessels. Graft of C3H foetal SI 9 days after implantation in a CBA mouse.
MGP x 150

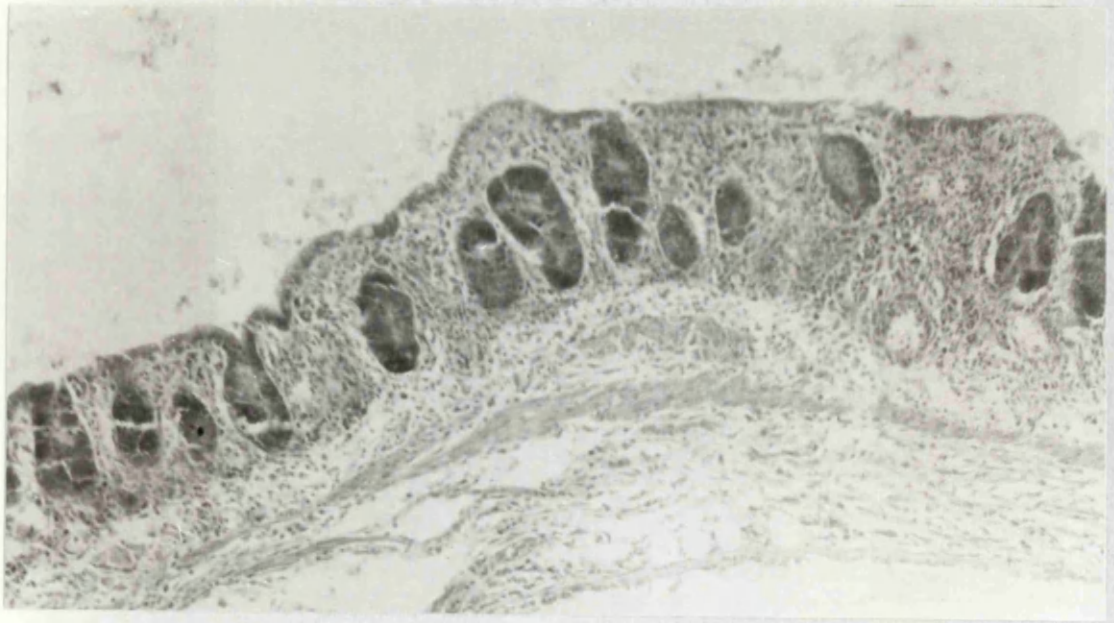

Fig. 9.2 Established rejection, grade Flat. The mucosal morphology is typical of subtotal villous atrophy. Graft of C3H foetal SI 10 days after implantation in a CBA mouse.
MGP x 150

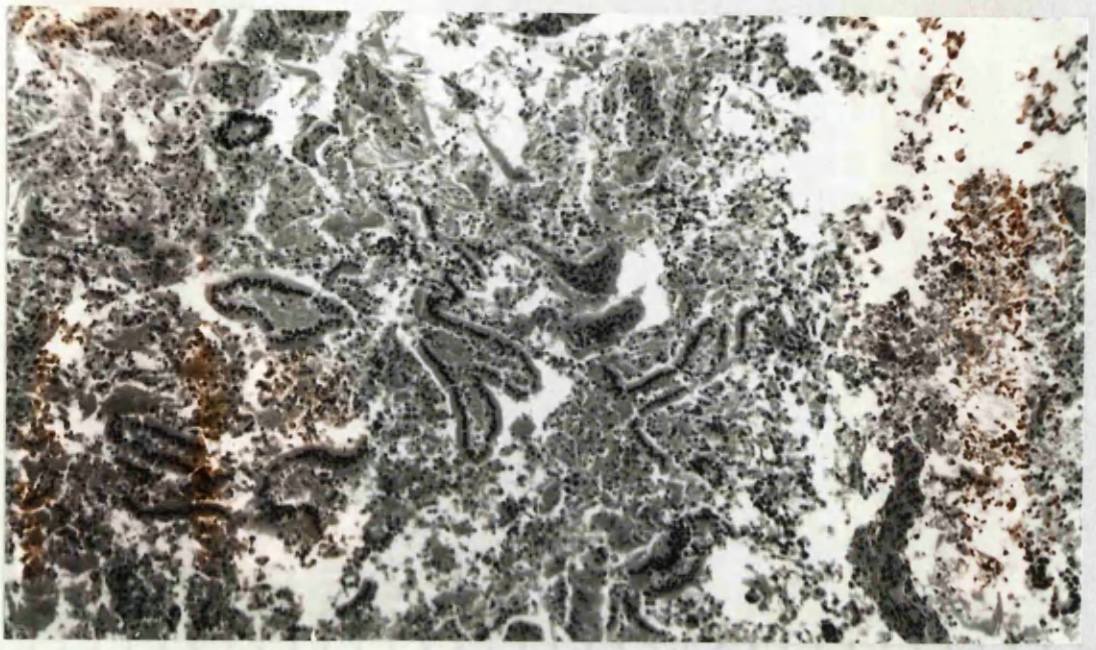

Fig. 9.3 Established rejection, with 'casts' of epithelial cells within the lumen of a graft. Graft of C3H foetal SI 15 days after implantation in a CBA mouse.
H & E x 159

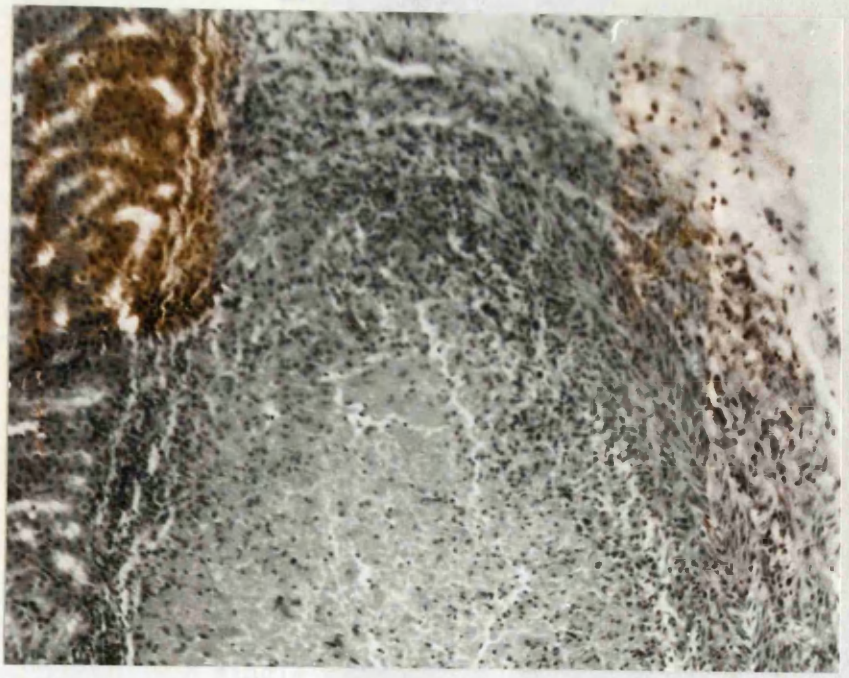

Fig. 9.4 Late rejection. No epithelium is present and the graft consists only of submucosa and muscle. Graft of CBA foetal SI 13 days after implantation in a BALB-c mouse.
H & E x 150

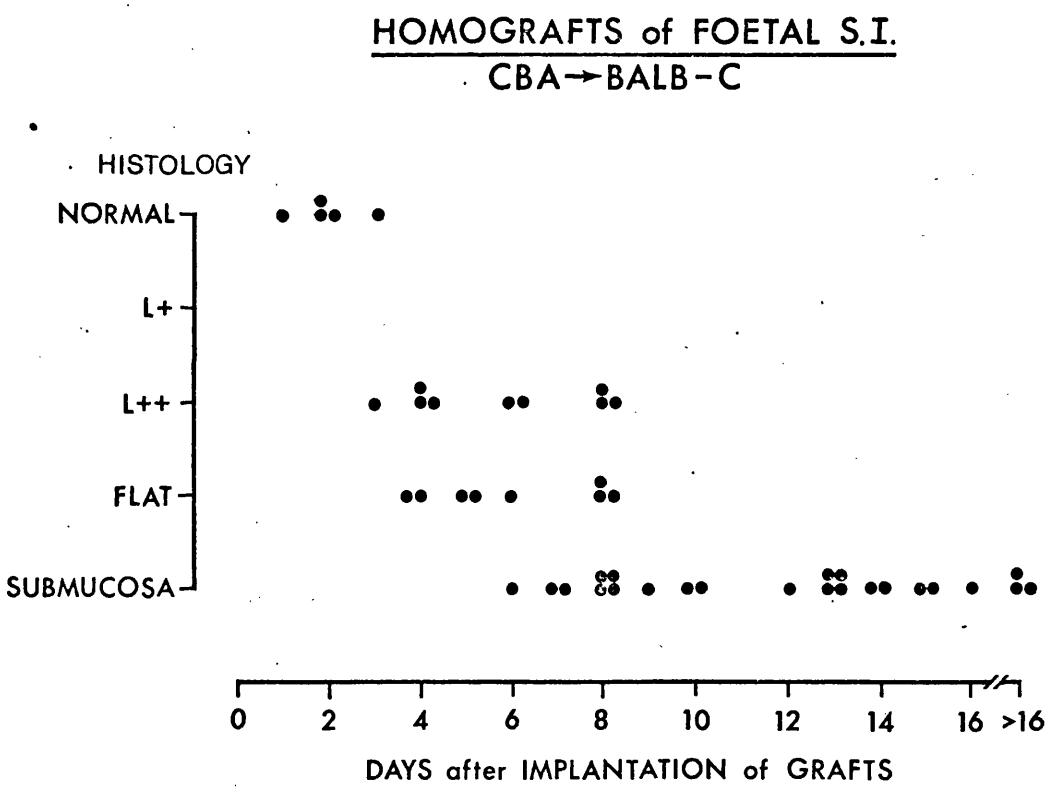

Fig. 9.5 Pathological trading of rejection of grafts of CBA foetal small intestine implanted in BALB-c mice. Each point represents the histology of a single graft. Gradings as described in text.

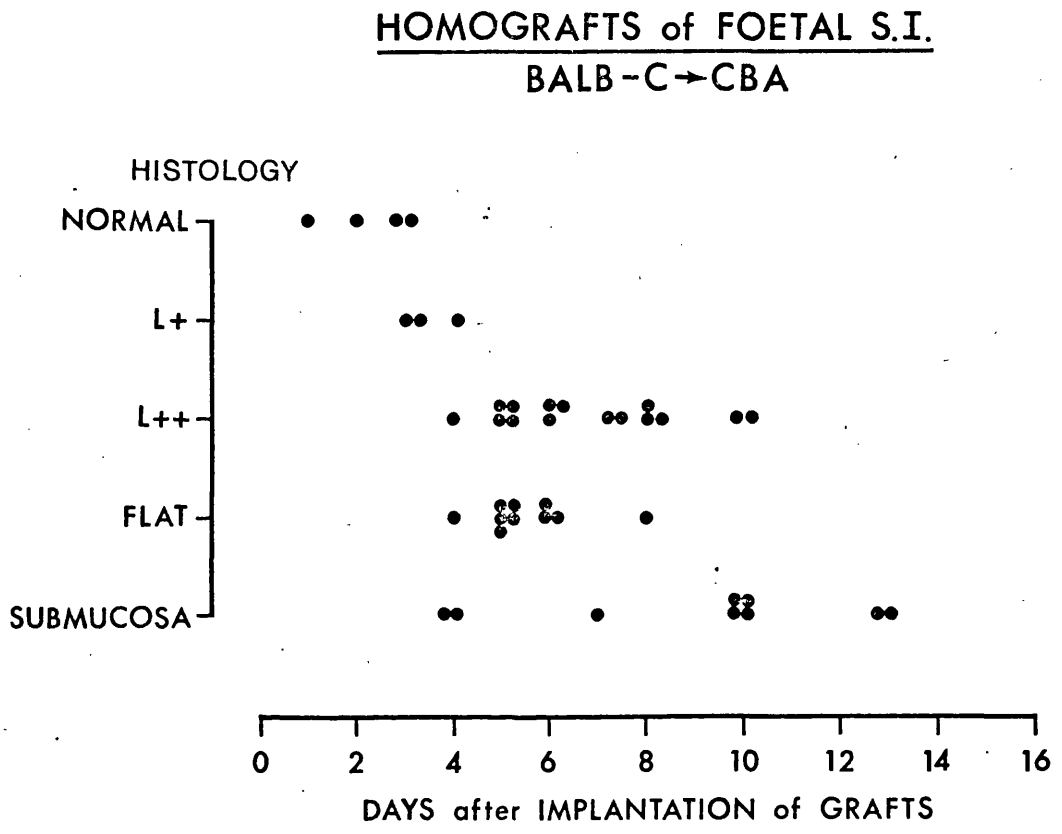

Fig. 9.6 Pathological grading of rejection of grafts of BALB-c foetal small intestine implanted in CBA mice. Each point represents the histology of a single graft. Gradings as described in text.

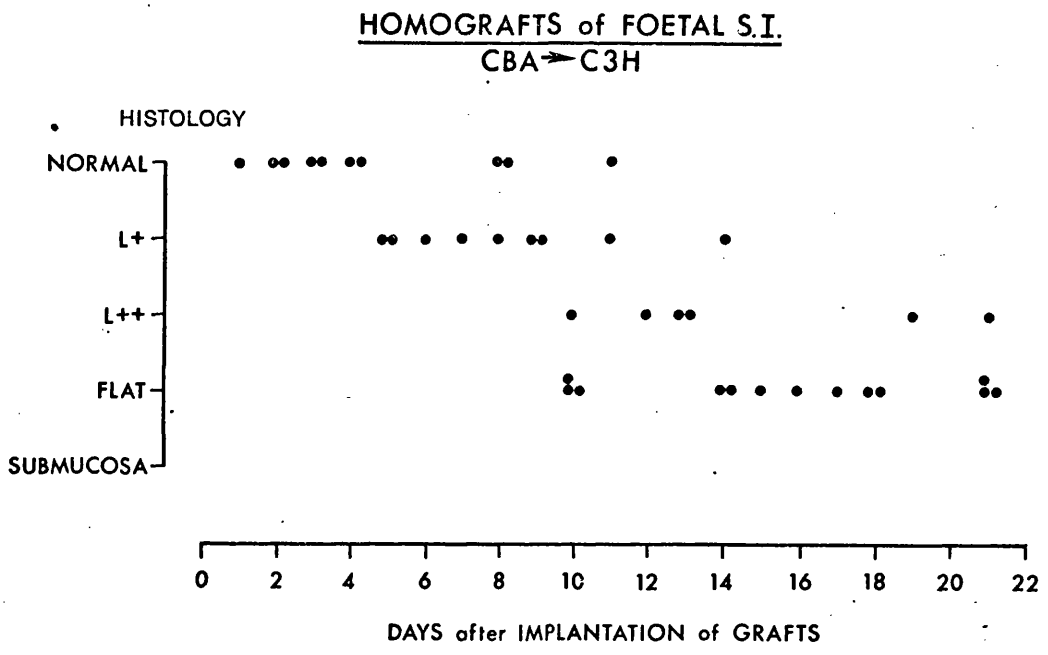

Fig. 9.7 Pathological grading of rejection of grafts of CBA foetal small intestine implanted in C3H mice. Each point represents the histology of a single graft. Gradings as described in text.

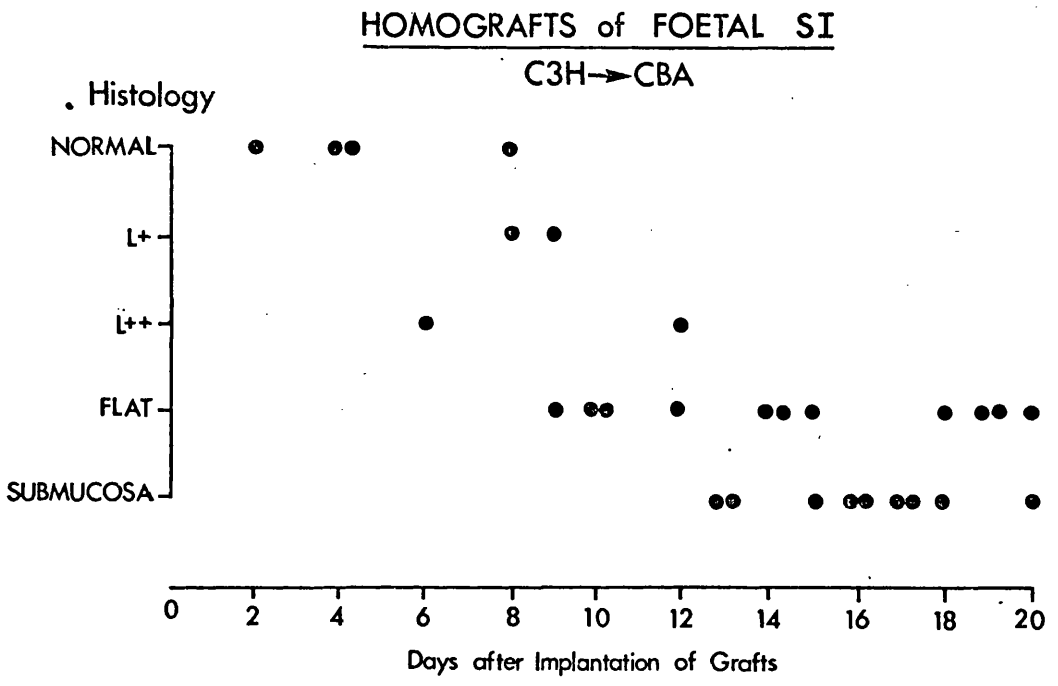

Fig. 9.8 Pathological grading of rejection of grafts of C3H foetal small intestine implanted in CBA mice. Each point represents the histology of a single graft. Gradings as described in text.

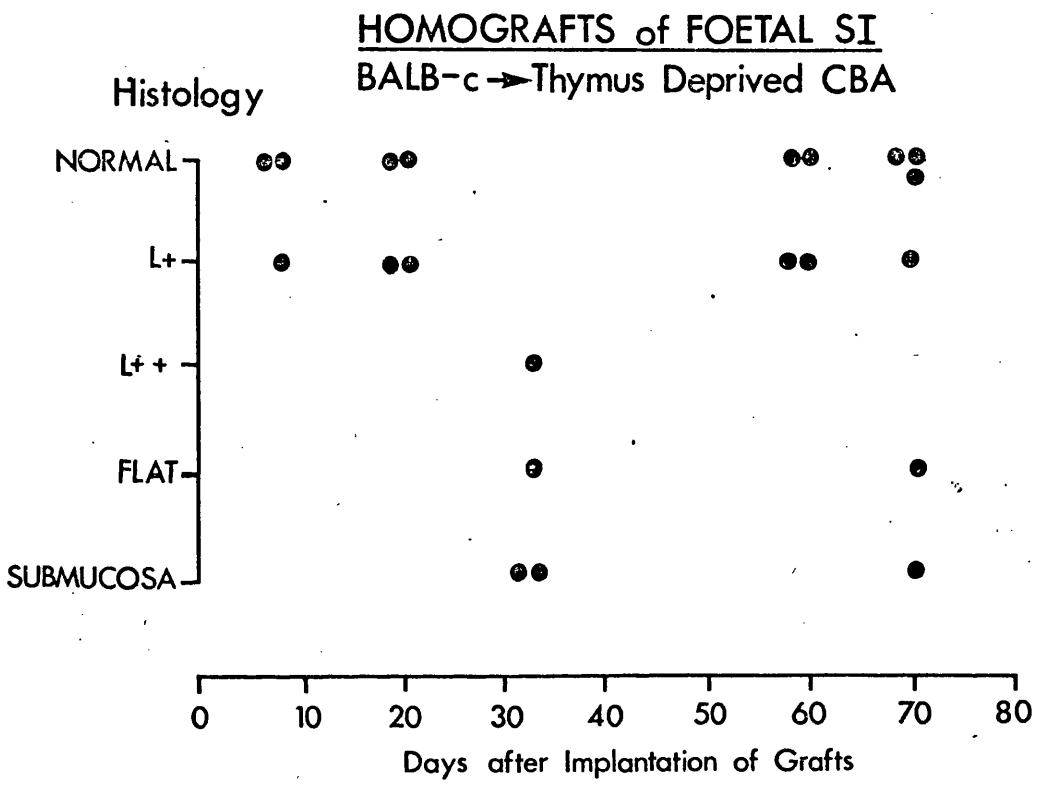

Fig. 9.9 Pathological grading of rejection of grafts of BALB-c foetal small intestine implanted in thymus-deprived CBA mice. Each point represents the histology of a single graft. Gradings as described in text.

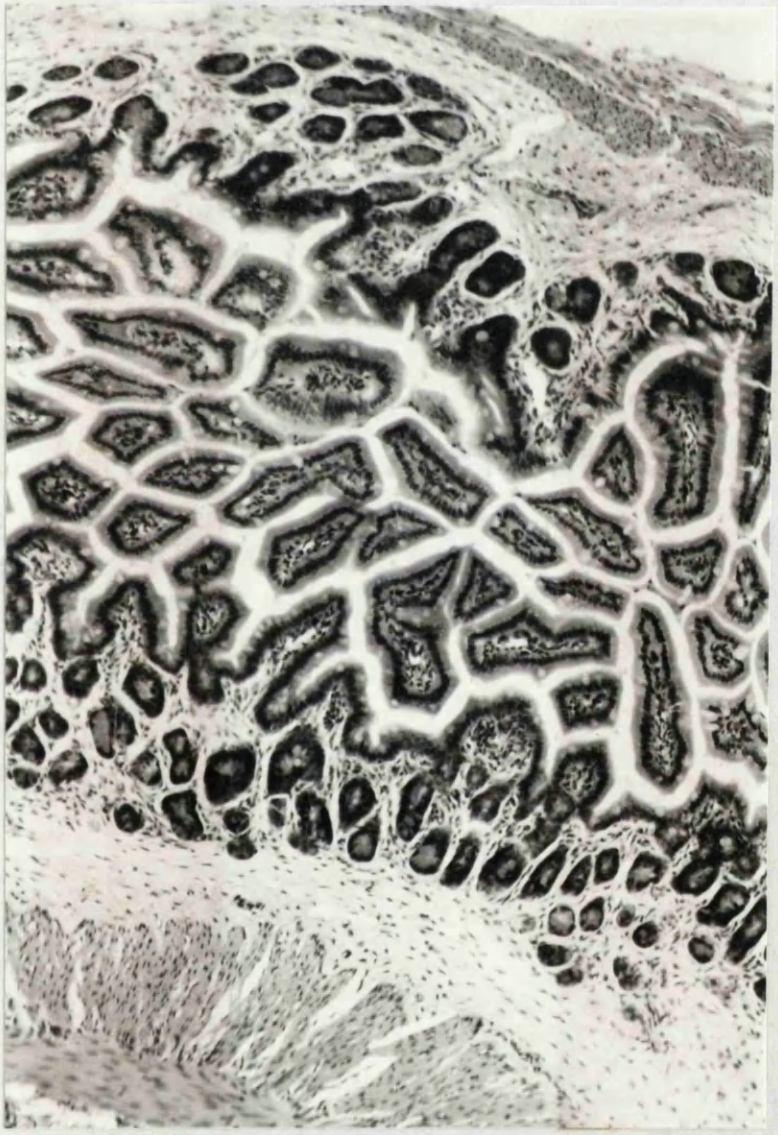

Fig. 9.10 Completely normal appearance of allograft of BALB-c foetal small intestine, 60 days after implantation in a thymus-deprived CBA mouse. H & E x 150

Chapter 10

General Discussion

The majority of nonepithelial cells within the small intestinal epithelium are medium sized lymphocytes, and although they may indent adjacent enterocytes, they are unequivocally intercellular in position. These findings agree with those of Meader and Landers (1967) and have recently been confirmed by other workers (Collan, 1972; Otto and Walke, 1972). The claim of Andrew (1965), that IE lymphocytes degenerate during their stay within the epithelium, has not been substantiated. Indeed, from a critical appraisal of the illustrations which were published it is clear that the intracellular inclusions which Andrew claimed to be degenerating lymphocytes have no features which allow a positive identification of lymphocytic origin.

The facts that IE lymphocytes are medium sized or large, and have irregular shapes, could be regarded as evidence that they are engaged in a local immunological response. However, when antigen-free mouse intestine was compared with normal human or mouse small intestine, no differences in the size of IE lymphocytes or in their morphology could be detected. The same is true of the intensely inflamed small intestine of patients with coeliac disease (Chapter 4). Otto and Walke (1972) have also found no ultrastructural features which allowed them to differentiate the IE lymphocytes of coeliac patients from those of normal individuals, even although the numbers of IE lymphocytes were greatly/

greatly increased in coeliac disease. Thus it seems likely that the fairly large size of IE lymphocytes is simply a reflection of the fact that lymphocytes which home to the intestinal villi are larger than the typical small lymphocytes which home to other lymphoid tissues.

Unfortunately, light and electron micrographs are unsuitable for assessment of dynamic phenomena such as motility of lymphocytes and the direction of migration across the basal lamina. In addition, the ultimate proof of an extra-intestinal origin of IE lymphocytes is still lacking, despite an extensive series of experiments involving intravenous infusion of isotopically labelled thymus, spleen and lymph node lymphocytes, none of which have been found in an intraepithelial site (Parrott and Ferguson, in press and Chapter 8). However, ^{3}H thymidine labelled mesenteric lymph node lymphoblasts enter the lamina propria, often at a site close to the basal lamina of the epithelium. Many lymphocytes can be seen in transit across this basement membrane (although labelled cells have not yet been observed to do so) and the IE lymphocytes are similar, in size and appearance, to those of the lamina propria. It seems likely that in further work on traffic of lymphoblasts it should be possible to strike on the right experimental conditions whereby labelled lymphocytes/

lymphocytes will be traced from blood, via the lamina propria, to the intraepithelial site.

The movements of lymphocytes within the epithelium must be considered in the context of the established kinetics of small intestinal epithelial cells. Enterocyte mitosis is confined to the crypts of Lieberkuhn; columns of enterocytes move from the crypts on to the sides of the villi and, in mice, are extruded from the villous tips at about 2 days after their formation (Leblond and Messnier, 1958). The irregular appearance of IE lymphocytes suggests that they also are motile. They are probably swept up the villi along with the sheets of enterocytes for they have no obvious sites of adhesion to the basement membrane. Within the moving epithelium they probably move around in the intercellular space, extending pseudopodia and perhaps sampling the environment by means of pinocytotic vesicles or caveolae. In this site they make contact with other lymphocytes (Chapter 4) and with processes of lamina propria macrophages (Collan, 1972). Finally, they may leave the epithelium by re-entering the lamina propria (Meader and Landers, 1967) or they may fall off into the intestinal lumen with the adjacent effete columnar cells (see Fig. 5.12). To date no function has been attributed to these free lymphocytes within the intestinal lumen and their lifespan within this environment/

environment has not been investigated. It is unlikely that they survive for long - certainly such free lymphocytes have not been observed within the lumena of the long-standing isografts of intestine studied in this thesis.

There are IE lymphocytes in the villi of rat, chicken and human foetuses, before exposure to any immunogens (Back 1972a; Fichtelius, 1969a; personal unpublished observations). They are also present in germ-free rats and mice and in established antigen-free grafts of mouse and rat intestine (Fichtelius, 1968; Chapters 4 and 5). Clearly the presence of IE lymphocytes does not correlate directly with the extent of antigen exposure, although local antigenic stimulation may increase their numbers.

The time course of appearance of lymphocytes in mouse intestine is interesting because lymphoid cells appear in the villi of normal mouse small intestine in the third week after birth, around the time of weaning, when the young mouse begins to sample foods other than milk. This is a time when there are profound changes in small intestinal enzymes (Moog, 1962; Arthur, 1968); in the mitotic rate of crypt cells and in enterocyte lifespan (O'Connor, 1966) and when there is cessation of pinocytosis and "closure" to the absorption of macromolecules such as immunoglobulins (Brambell/

(Brambell, 1966, 1970). All of these seem appropriate adaptations to changes in amount and type of food in the weanling intestine (Sunshine, 1971), yet although closure, enzyme patterns and cell turnover times can be influenced by cortisone treatment or by alterations in diet (reviewed by Koldovsky, 1969; Brambell, 1970), the factors which initiate these changes in the normal situation have still to be defined.

In my studies of the maturation of isografts of intestine, two of these weanling changes correlate with the lymphocyte studies. Pinocytosis has only been observed in antigen-free grafts of less than 17 days duration (P G Toner, unpublished). Also, sequential changes in the brush border enzymes, sucrase and lactase, occurred in grafts of small intestine at the same time as in normally sited intestine (Ferguson et al, 1973). Thus, for these two properties at least, the capacity for maturation from pre-weaning to post-weaning is already present in foetal small intestine at the time of grafting.

The penetration by lymphocytes of the weanling small intestine could be for several reasons. The appropriate lymphocytes may be released into the vascular compartment around this time; their appearance in the intestinal villi may be the result of the presence of food or microbial antigens within the gut lumen; the small intestine may only become receptive to the entry of lymphocytes/

lymphocytes around this time; or a combination of the above factors may be involved. Grafts of small intestine are implanted in adult hosts, whose normally sited small intestinal villi contain many lymphoid cells. However, it is only after the grafted tissues have reached an age equivalent to the usual time of weaning (2-3 weeks) that graft villi become populated by lymphocytes and plasma cells. Thus, in the same way that grafts have an intrinsic capacity for maturation of enzyme patterns, they seem to have an intrinsic time-based mechanism whereby they only become receptive to the entry of certain types of lymphocytes at 2-3 weeks after the time of birth. Further experiments are required to identify just what aspect of local tissue maturation is essential for the onset of lymphoid cell infiltration of villi. Possibilities include changes in the endothelium or basement membranes of the capillaries or venules of the villi, changes in the ground substance of the lamina propria, or the onset of local secretion of hormones by the intraepithelial endocrine cells.

The experiments described in Chapters 5 and 6 show that the IE lymphocytes do not form a uniform population, but that there are thymus-dependent and thymus-independent cells, whose numbers/

numbers and relative proportions vary according to the age of the small intestinal tissue, and to the nature and degree of antigenic exposure via the lumen. Indeed, perhaps the most striking finding has been that in the absence of intraluminal stimulation by the antigens normally present in the gut there are profound effects on the IE lymphocytes, whose numbers were dramatically reduced (in antigen-free grafts) when compared with normally-sited small intestine of the same "post-natal age" or with the host animal's own intestine. Grafts of gut implanted under the kidney capsule are not, of course, normal, but their growth and development closely parallel normal gut and it seems justifiable to ascribe the differences in lymphocyte populations to immunological processes rather than to differences in vascular or nervous connections, although these cannot be ruled out entirely.

It is interesting that there were similar numbers of IE lymphocytes in established antigen-free isografts of small intestine in CBA mice and in the normally-sited small intestine of germ-free mice of the C3H strain. This coincidence in results may be merely fortuitous. However, it may indicate that the antigens within the lumen of germ-free mouse intestine play little part in the regulation of the numbers of IE lymphocytes and of lamina propria/

propria lymphoid cells. This would be in contrast to the influence of these intraluminal antigens on Peyer's patches, for the Peyer's patches of germ-free mice were much larger than the Peyer's patches of antigen-free grafts.

These findings of low IE lymphocyte counts in germ-free mice differ from those of Fichtelius, who reported that there was no difference in the numbers of IE lymphocytes when germ-free and conventionally reared rats were compared (Fichtelius, 1968). However, he studied only 4 germ-free rats and it may be that he would have found significant differences if he had used a larger series. Nevertheless, my own results could be criticised because I did not have a group of conventionally reared C3H mice from Dr. Salomon's laboratory with which to compare the germ-free C3H from the same source.

The comparisons between normal, germ-free and antigen-free gut are important because in germ-free mice there is profound reduction of antigenic stimulation, not only via the gut lumen but also systemically. In contrast, isografts of gut are deprived of intraluminal antigenic stimulation but grow in fully mature, conventionally reared and fed, mice, many of which harbour parasites and pathogenic microorganisms. It is already known that macromolecules can be traced from the bloodstream to the extracellular fluid of the lamina propria and even to the inter-

cellular spaces of normal intestinal epithelium (Dobbins et al, 1968). There seems no reason why such macromolecules (eg derived from foods) and other potential immunogens (eg endotoxins) should not gain access to the lamina propria of grafts of intestine. Yet the low numbers of IE lymphocytes and plasma cells in grafted intestine suggests that their presence in the villi relates to intraluminal antigens rather than antigen elsewhere in the animal. This seems also to be true for Peyer's patches of grafted intestine, which are tiny and lack germinal centres, even though germinal centres are present in almost all the other peripheral lymphoid tissues of the host.

There are at least two ways in which the presence of intraluminal antigen may influence the numbers of IE lymphocytes. Local antigen within the small intestine may attract to the villi larger numbers of lymphoblasts. Alternatively, there may be a uniform rate of homing of lymphoblasts to the gut, irrespective of the presence of intraluminal antigens, as has been demonstrated in recent experiments on the homing of rat lymphoblasts (Moore and Hall, 1972). If the traffic of lymphoblasts is constant, increased numbers of IE lymphocytes (and of lamina propria lymphoid cells) could result from an effect of antigen, prolonging their stay in the villi. Alternatively, antigen could induce further cell division, or increase their rate of mitosis. We have found that/

that about 5% of normal small intestinal IE lymphocytes will incorporate ^{3}H thymidine after an intraperitoneal injection of 25 uc ^{3}H thymidine (Ferguson and Parrott, unpublished). This seems to be uninfluenced by the presence of intraluminal antigen since a similar rate of ^{3}H thymidine incorporation was obtained for IE lymphocytes of antigen-free grafts. Thus, differences in the incidence of IE lymphocyte mitosis cannot be the only explanation for the antigen-related variation in numbers of IE lymphocytes.

I have been able to study the small intestine in thymus-deprived and intact mice, prepared and reared in a variety of ways, and thereby have demonstrated that there are both thymus-dependent and thymus-independent IE lymphocytes in the small intestine. I found that at the age of 6 weeks, neonatally thymectomised mice had only one-third as many IE lymphocytes as their untreated littermates. Similar findings have been made in mice and chickens (Fichtelius et al, 1968b; Back, 1970a). Whereas the differences between thymectomised and intact conventionally reared mice became less with age, this was not so for germ-free mice. In the group studied, aged 6 months, there were still significantly/

significantly fewer IE lymphocytes in the neonatally thymectomised mice when compared with their littermates. Somewhat surprisingly, a significant reduction in IE lymphocyte counts was not apparent in grafts implanted in thymus-deprived mice, even though the hosts had low IE lymphocyte counts in their normally sited small intestine.

The differences between IE lymphocyte counts in neonatally thymectomised and normal mice, although minor in comparison with the effects of antigen, have considerable theoretical importance for they demonstrate that, as in the Peyer's patches, both thymus-dependent and thymus-independent IE lymphocytes are present. The facts of their dual nature and their ability to respond to antigen argue against the theory that they constitute a primary lymphoid organ or bursa equivalent.

Of the various groups of thymus-deprived and normal mice which have been compared, the most profound differences have been found in young mice, and in germ-free animals - ie it is in these situations that T cells form a relatively greater proportion of the IE lymphocytes. In this property the composition of the IE lymphocytes corresponds with that of the Peyer's patches (Chapters 2 and 7). These findings may indicate that thymus-dependent lymphocytes in the villi of weanling and germ-free mice are/

are involved in the immune responses to non-living, dietary antigens, whereas immune responses to commensal gut bacteria are mainly mediated by thymus-independent, B lymphocytes.

Although the experiments discussed above indicated that IE lymphocyte populations have attributes of thymus-dependence and thymus-independence, the lymphocyte migration studies (Chapter 8) show clearly that lymphocytes of the intestinal villi must represent special categories of T and B cells. The only labelled lymphocytes which could be traced to the villi were ^{3}H thymidine labelled lymphocytes from mesenteric lymph node of mice which had been either newly stimulated with food antigens or which were infected with intestinal parasites. Thus, their mesenteric lymph nodes probably contained many cells specifically orientated towards the gut, or gut antigens. However, these primed cells homed to gut tissue even in the absence of intra-luminal gut antigens (ie to grafts) and within the limits of assessment from sections of autoradiograph the numbers of injected cells found in grafts were the same as in normally sited gut - findings which agree with those of Hall and his co-workers in similar experiments in rats (Halstead and Hall, 1972; Moore/

Moore and Hall, 1972). This quite distinct group of cells which migrated to the lamina propria of mouse gut resembled closely, in morphological and "source" characteristics, the cells which other workers have found to home to the lamina propria. They are large lymphocytes, they label in vitro and in vivo with ^{3}H thymidine, and are found in thoracic duct lymph or in the mesenteric lymph node (Gowans and Knight, 1964; Delorme et al, 1969; Griscelli et al, 1969; Hall et al, 1972). The source of these lymphoblasts seems to be important, for in my experiments ^{3}H thymidine-labelled large lymphoid cells taken from sources other than mesenteric node did not home to the gut.

Unfortunately there is, as yet, no proof that the same cells which home to the lamina propria also penetrate the epithelium or are the progeniton of the IE lymphocytes. Goldschneider and McGregor mentioned that they traced some rat lymph immunoblasts to an intraepithelial site, but they did not illustrate this point and have not published further data (Goldschneider and McGregor, 1968a). Hall and his co-workers have carried out many experiments similar to Goldschneider's and Dr. Hall has quite firmly stated that on examination of his own autoradiographs (including electron microscopic autoradiographs) all the labelled cells can definitely be identified as lying within the lamina propria (Hall, personal communication). Obviously this point deserves further/

further study for there is nothing to suggest that the IE lymphocytes develop from epithelial cells; they must have entered the epithelium from the lamina propria or directly from the subepithelial blood vessels, and it should be possible to demonstrate this by standard cell migration study techniques.

Migration of lymphocytes to the thymus-dependent and nodular areas of Peyer's patches was as would be predicted from the established homing patterns of thymus cells, B lymphocytes and of normal spleen cells (Parrott and de Sousa, 1969; 1971). Thymocytes homed to thymus-dependent areas of Peyer's patches, spleen cells from B mice were found mainly in thymus-independent, nodular areas and spleen cells from normal mice were found in all areas of Peyer's patches. Moreover, these lymphocytes homed to Peyer's patches of grafts as well as of normal gut, but although they were occasionally found in the lamina propria of villi adjacent to Peyer's patches they were never found in other small intestinal villi.

There appear to be several clear cut differences between Peyer's patch and small intestinal IE lymphocytes. Firstly, Peyer's patches are present in foetal and newborn mice and in gut grafts, two weeks before IE lymphocytes. One could argue that/

that in the neonate there are insufficient lymphoid cells in the circulation for them to appear in the gut wall, but this would not apply to the grafts. It seems that the gut epithelium only becomes permeable to its normal population of lymphocytes in the third week of life, whereas, even in the newborn, the tiny Peyer's patches contain post-capillary venules and so can be populated by lymphocytes via this well-established route. In the course of rejection of allografts of small intestine, the post-capillary venules seem also to provide a route of entry of allogeneic lymphocytes, although lymphocytes are also found in the lamina propria, remote from Peyer's patches, early in the rejection process (Chapter 9).

Secondly, there are somewhat different effects of intraluminal antigen deprivation on Peyer's patches and IE lymphocytes. Peyer's patches of germ-free mice are intermediate in size between those of antigen-free and normal gut, whereas IE lymphocyte counts were similar in the germ-free and antigen-free situations. It is tentatively concluded that, in mice, the numbers of IE lymphocytes seem to be directly related to the extent of microbial contamination of the small intestine, whereas the size of Peyer's patches is influenced by intraluminal food antigens and killed microorganisms as well as by live microbes.

Finally, /

Finally, as discussed extensively above, completely different populations of isotopically labelled lymphocytes have been found to home to the Peyer's patches and to the lamina propria.

One further point which deserves mention is the category into which one should place the intraepithelial lymphocytes which lie between the epithelial cells overlying Peyer's patches. These cells seem to behave as Peyer's patch cells, rather than as typical small intestinal IE lymphocytes, for example they are present in the epithelium overlying Peyer's patches in the mouse foetus.

The morphological features of rejection of allografts of small intestine have provided further information regarding the nature and possible functions of intestinal lymphocytes. The early penetration of allografts by lymphocytes is important evidence that the renal subcapsular site is in no way immunologically privileged. Lymphocytes have no difficulty in entering allografts on the third day after implantation and this renders even more significant the two week post-natal delay before entry of lymphocytes into the villi of isografts.

The/

The experiments described in Chapter 9 show that even though the small intestine is a lymphoid organ, its rejection resembles that of skin in that it is influenced by the degree of histoincompatibility between donor and host (Billingham et al, 1954; Medawar, 1963) and by thymus-deprivation of the host (Miller et al, 1963; Cross et al, 1964). Some longstanding allografts in thymus-deprived hosts had completely normal appearances, and had been populated by plasma cells and lymphocytes to the same extent as isografts of the same age. This is further evidence that the majority of intestinal villous lymphocytes and plasma cells are thymus-independent, B cells, and that these B cells do not have the capacity to initiate allograft rejection.

The histological similarities between established rejection and the "villous atrophy" of coeliac disease (Hourihane, 1966; Cappell and Anderson, 1971) are striking, although there are minor differences between these two conditions. In rejection the epithelial cells often retain a normal appearance until a late stage; also there is less lymphocyte infiltration of the villous epithelium in rejection than in coeliac disease. Nevertheless, my findings, together with the reported histopathology of allograft rejection of dog small intestine (Holmes, et al, 1971) and the report of villous atrophy as the intestinal lesion of graft-versus-host disease in dogs (Reilly and Kirsner, 1965) suggest that villous/

villous atrophy can be brought about by activated T cells, although these findings are equally consistent with the notion that thymus-dependent antibody is responsible for the lesion. This could mean that in the intestinal lesion of coeliac disease there are accumulations of activated T lymphocytes. These could be directed against the intestinal tissues per se, but more likely are directed against food antigens which have crossed the intestinal epithelial cell barrier.

The clinical relevance of investigation of the IE lymphocytes has been demonstrated by the results of quantitation of these cells in jejunal biopsies. Only in patients with untreated coeliac disease were the IE lymphocyte counts consistently high - a quantitative confirmation of Hourihane's qualitative description of increased lymphocyte infiltration of the jejunal epithelium in coeliac disease (Hourihane, 1966), and results which have subsequently been confirmed by other workers (Otto and Walke, 1972; Holmes et al, 1973). IE lymphocyte counts fall when coeliac patients follow a gluten-free diet (Chapter 4, and Kumar et al, 1973; Holmes et al, 1973). These facts, together with the morphological resemblance/

resemblance between coeliac disease and intestinal allograft rejection, suggest that cell-mediated immune reactions (possibly to gluten) are instrumental in producing the small intestinal lesion of villous atrophy.

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Intestinal Immunity. The role of intraepithelial lymphocytes.

Anne Ferguson.

Summary

The mammalian small intestine is lined by a columnar epithelium, the orderly arrangement of which is interrupted by the presence within it of many intraepithelial lymphocytes. In this thesis the nature and possible functions of these intraepithelial (IE) lymphocytes have been investigated in two ways, by morphological examination and quantitation of human jejunal IE lymphocytes, and by a series of experiments in mice.

Human small intestinal IE lymphocytes resemble their counterparts in other species, both in their light microscopic and ultrastructural features. They are basally situated in the epithelium, lie between and not within the epithelial cells, and are frequently seen crossing the epithelial basement membrane. IE lymphocytes were quantitated in 200 routinely processed jejunal biopsy specimens, and the normal range of values has been established as 6-40 IE lymphocytes per 100 villous epithelial cells. Only in biopsies from patients with coeliac disease were the IE lymphocyte counts consistently higher than normal.

The design and interpretation of experiments in mice were facilitated by the use of "antigen-free" loops of small intestine. These were prepared by transplanting foetal small intestine under the kidney capsules of adult mice of the same strain. Such grafts healed in, grew and had normal morphological appearances as assessed by light and electron microscopy, although they had never been exposed to intraluminal antigens.

Thymus-dependent and thymus-independent IE lymphocytes, and IE lymphocytes specifically related to antigens within the gut, were identified by examination of small intestine from normal mice, germ-free mice and antigen-free grafts. In all these situations, normal and thymus-deprived mice were compared. These experiments showed that in the absence of intraluminal stimulation by antigens normally present in the gut there were profound effects upon all the intestinal lymphoid tissues.

Antigen deprivation had a profound effect on the IE lymphocytes, whose numbers were considerably reduced in germ-free and antigen-free gut when compared with normally sited gut of the same age. In addition, the consistent findings of low IE lymphocyte counts in thymus deprived animals demonstrated that there were both thymus-dependent and thymus-independent IE lymphocytes; thymus-dependent lymphocytes formed a relatively greater proportion of the total in young animals and in germ-free/

germ-free mice. Antigen deprivation had similar effects on Peyer's patches, which contained both thymus-dependent and thymus-independent lymphocytes, were small in germ-free and antigen-free gut, and which lacked germinal centres in both of these situations. From these observations, it has been tentatively concluded that, in the normal small intestine, immune responses to commensal gut bacteria may be mediated by thymus-independent lymphoid tissue, whereas in young or germ-free mice, thymus-dependent lymphocytes are involved in the immune responses to non-living, dietary antigens.

The factors which determine the migration of lymphoid cells to the lamina propria or Peyer's patches of mouse small intestine were investigated by autoradiographic tracing of intravenously injected spleen, thymus and lymph node cells. The numbers of labelled cells found in antigen-free grafts were compared with the numbers in normally sited gut. Thymus, normal spleen and B spleen lymphocytes, labelled with ^{3}H-adenosine, were confined to Peyer's patches in normal and grafted gut; ^{3}H-thymidine labelled lymphoblasts from the mesenteric nodes of young mice, and mice infected with Nippostrongylus brasiliensis, were found in the lamina propria of both normal and grafted gut, although none of these labelled lymphocytes were traced to the intraepithelial site. These results indicated that the lymphocytes of intestinal villi represent special categories of T and B cells, /

cells, specifically orientated towards the gut, although not (in these experiments) towards antigens within the gut lumen.

The technique of gut grafting was also applicable to allografts, and from morphological examination of such grafts the features and time course of rejection of mouse small intestine have been determined. The earliest sign of rejection was the appearance of lymphocytes in the graft lamina propria. Later there was extensive lymphocyte infiltration of the full thickness of the intestine, and many tissues had the general mucosal morphology of partial or subtotal villous atrophy. Later there was complete destruction of the mucosa and the remnants of the grafts consisted of smooth muscle heavily infiltrated with lymphocytes and plasma cells. These same events occurred in sequence in all the strain combinations studied but the time course of rejection varied with histocompatibility differences between donor and host strains. Thymus-deprived mice showed profoundly impaired ability to reject allografts.

The findings of increased numbers of IE lymphocytes in coeliac disease, and of histological similarities between established rejection and coeliac disease, suggest that villous atrophy may result from the presence of activated lymphocytes in the small intestine. These could be directed against the intestinal tissues per/

per se, but more likely are directed against food antigens which have crossed the intestinal epithelial cell barrier.