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The influence of gut function on lymphoid cell populations in the intestinal mucosa of lambs

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Summary. The number and type of lymphoid cells in the intestinal mucosa of lambs change during the first weeks after birth. The influence of gut function on these changes was examined by comparing the evolution of lymphoid cell populations in normal ileum with that in lengths of ileum which had been isolated surgically from the functional intestinal tract of the lamb before birth. The isolated lengths of ileum had a normal blood and nerve supply and they remained healthy throughout a period of at least 2 years, although they did not have a normal histological development. In comparison with normal ileum, the villi of the isolated ileal segments were much smaller and there were many fewer intraepithelial lymphocytes; the lamina propria had signficantly fewer lymphocytes than the functional ileum and only a few plasma cells. When isolated ileal segments were reconnected into the intestinal tract after having been isolated from it for 1–3 months, the histology of the mucosa reverted to that of the normal gut, with the same number and types of lymphoid cells. Radiolabelled lymphoblasts collected from intestinal lymph and injected intravenously accumulated to only a small extent in isolated segments of ileum compared with either the normal or the reconnected segments of ileum. This suggested that the paucity of lymphocytes in the mucosa of the isolated segments was due to a reduced extravasation of these cells there. The influence which the gut contents exert on the lymphoid cell population in the mucosa is probably associated with antigenic stimulation but may also be related to other factors concerned in the normal digestive functions of the gut.

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INTRODUCTION

The alimentary canal provides a large absorbing surface for molecules derived from the digestive process; some of the molecules that are absorbed are immunogenic (Warshaw, Walker & Isselbacher, 1974). As a consequence, the gut is a major site of entry of immunogenic material into the body, and associated with it, there is an extensive lymphoid apparatus comprising the mesenteric lymph nodes, the Peyer's patches and a diffuse population of lymphoid cells in the mucosa that are part of the migrating lymphocyte population passing between the blood, the tissues and the lymph. Once an animal is born, antigen is continually present in the gut lumen, and this makes it difficult to establish the extent to which the development of the gut-associated lymphoid tissue reflects a normal growth pattern or the response to a perennial antigenic challenge. However, in species with epitheliochorial placentation, such as the sheep, the foetus

has no contact with foreign antigen and while *in utero* it is essentially agammaglobulinaemic. Thus lymphoid development in foetal lambs proceeds independently of foreign antigen or immunoglobulin (Fahey & Morris, 1978).

We have examined the development of the diffuse lymphatic tissue in the intestinal mucosa of the foetal lamb throughout gestation and following birth, when there is an abrupt change in many aspects of gut function and when foreign antigen enters the intestinal tract for the first time. The influence of the products of digestion on the histological development of the mucosa was studied in lambs in which segments of ileum had been isolated from the intestinal tract. These segments had normal nerve and vascular connections but had no involvement in the reactions associated with normal digestion.

MATERIALS AND METHODS

Animals and surgical procedures

Merino, Border Leicester, and crossbred ewes were mated to Merino or Dorset rams fitted with a harness and marking crayon. The service date was noted so that the exact gestational age of the foetus could be determined.

Isolation of segments of ileum in foetal lambs. Operations were done on foetal sheep at 116–141 days gestation as described by Smeaton *et al.* (1969). The peritoneal cavity of the foetus was opened on the right side and the terminal part of the ileum located and mobilized. A 30-cm length of ileum was isolated by transecting the intestine about 20 cm and 50 cm from the ileocaecal junction. Each of the four open ends of intestine were closed by sutures and the ends of the intestine proximal and distal to the isolated ileum were positioned so that they were isoperistaltic and overlapped by about 3 cm. A side-to-side anastomosis (1–2 cm long) was used to re-establish the continuity of the intestine. The mesentery was left intact so that the vascular and nerve connections to the isolated ileal segment were not affected. The foetal intestines were returned to the peritoneal cavity and the abdominal wall was closed with interrrupted sutures. The incisions in the foetal and maternal tissues were sutured closed as described elsewhere (Smeaton *et al.*, 1969).

Reconnection of the isolated ileal segment into the intestinal tract. This operation was done in lambs after birth, between 1 and 4 months after the ileal segment had originally been isolated. A laparotomy was done and the isolated length of ileum was divided into two parts by transecting it about 10 cm from the proximal end, and each part was closed with sutures. The functional intestine was then divided about 30 cm from the ileocaecal junction proximal to the previous side-to-side anastomosis and the ends closed. The 10-cm length of isolated ileum was positioned next to the closed ends of the divided functional intestine and connected to its two ends by side-to-side anastomoses. The peritoneal cavity was closed and the muscle and skin layers sutured.

Intestinal biopsy. The proximal end of the isolated ileal segment was mobilized through a laparotomy incision. This end was originally continuous with the normal ileum at the proximal end of the anastomosis; biopsy tissue was taken from these two regions.

Collection of intestinal lymph

The main intestinal lymphatic duct was cannulated in lambs following the method described by Lascelles & Morris (1961). The cell concentration in lymph was determined with a model Fn Coulter Counter (Coulter Electronics, Dunstable, U.K.).

Labelling cells with [^{3}H]-thymidine

Lymph was collected over periods of 10–18 hr and centrifuged for 5–10 min at 1000 r.p.m.; the cells were resuspended at a concentration of 10^9/ml in Hanks's balanced salt solution containing 10% foetal calf serum and 20 μCi/ml [^{3}H]-thymidine (5 Ci/mmol; Radiochemical Centre, Amersham, U.K.). The cells were incubated for 1 hr at 37°, then washed four times in 10 ml of [^{3}H]-thymidine-free medium. The washed cells were made up to a concentration of 10^9/ml and injected intravenously into the lamb from which they had been collected. Smears of the cells used for injection were fixed for 15 min in methanol and prepared for autoradiography.

Histology and autoradiography

Tissue samples were fixed overnight in either cold Carnoy's fluid or in cold, freshly prepared, 3·5% formaldehyde in phosphate-buffered saline (pH 7·2), and embedded in either glycol methacrylate (JB-4 embedding kit, Polysciences Inc., Warrington, U.S.A.) or in paraffin. Methacrylate embedded tissue

sections (1 μm) were stained with Nocht's azure-eosin at pH 5·2 (Bennett *et al.*, 1976) and those embedded in paraffin (7 μm) were stained with haematoxylin-eosin or methyl-green pyronin (Luna, 1968).

Autoradiography was done with unstained methacrylate or paraffin sections and with smears of lymph cells. The slides were dipped in diluted (1:1) Ilford K5 emulsion, dried, stored at 4° in light-tight boxes, and exposed for periods varying from 5–40 days before development.

Measurement of radioactivity

The distribution of radioactivity in tissues was measured 40 hr after the intravenous injection of [^{3}H]-thymidine labelled cells. Tissue samples were weighed, then dried for 2–3 days, before being combusted in a model 306 Packard Tricarb Oxidizer and mixed with scintillation fluid ('Monophase-40', Packard); the samples were counted in a Packard model 3320 Liquid Scintillation Spectrometer.

RESULTS

A total of 27 foetuses were prepared successfully with isolated lengths of ileum. These lambs were born normally and survived and grew well. The isolated ileal segments remained healthy in appearance throughout the 2-year period of the experiment (Fig. 1). The distal part of the segment became distended

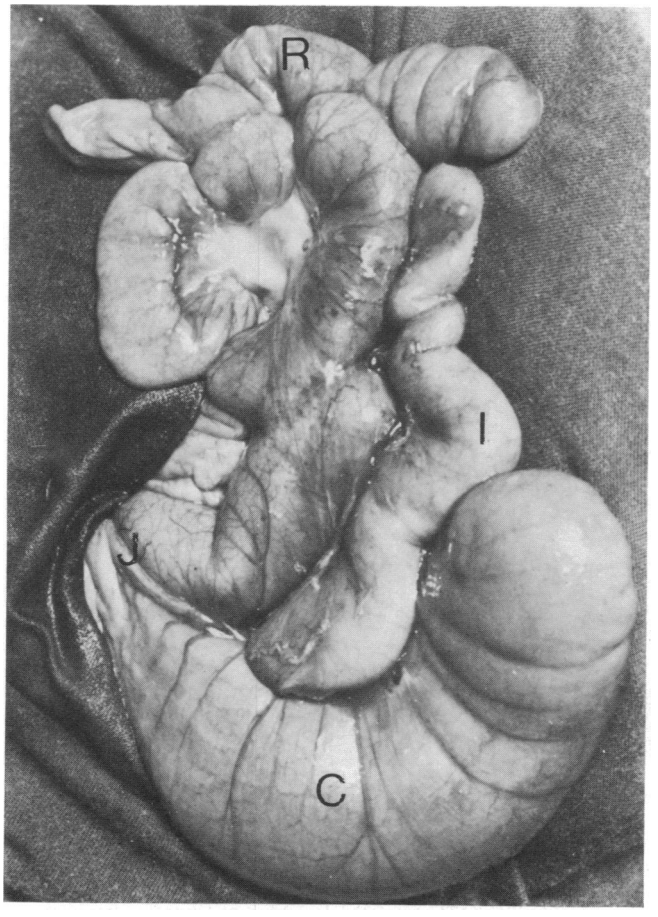

Figure 1. Isolated (I) and reconnected (R) segments of ileum in a 4-month-old lamb. The segment had been isolated from the ileum at 128 days gestation, and part of the isolated ileum was then reconnected into the intestinal tract 1 month after birth. The caecum (C) and ileo-caecal junction (J) are also seen in the photograph. The histology of biopsies from the isolated ileum and reconnected ileum is shown in Fig. 2.

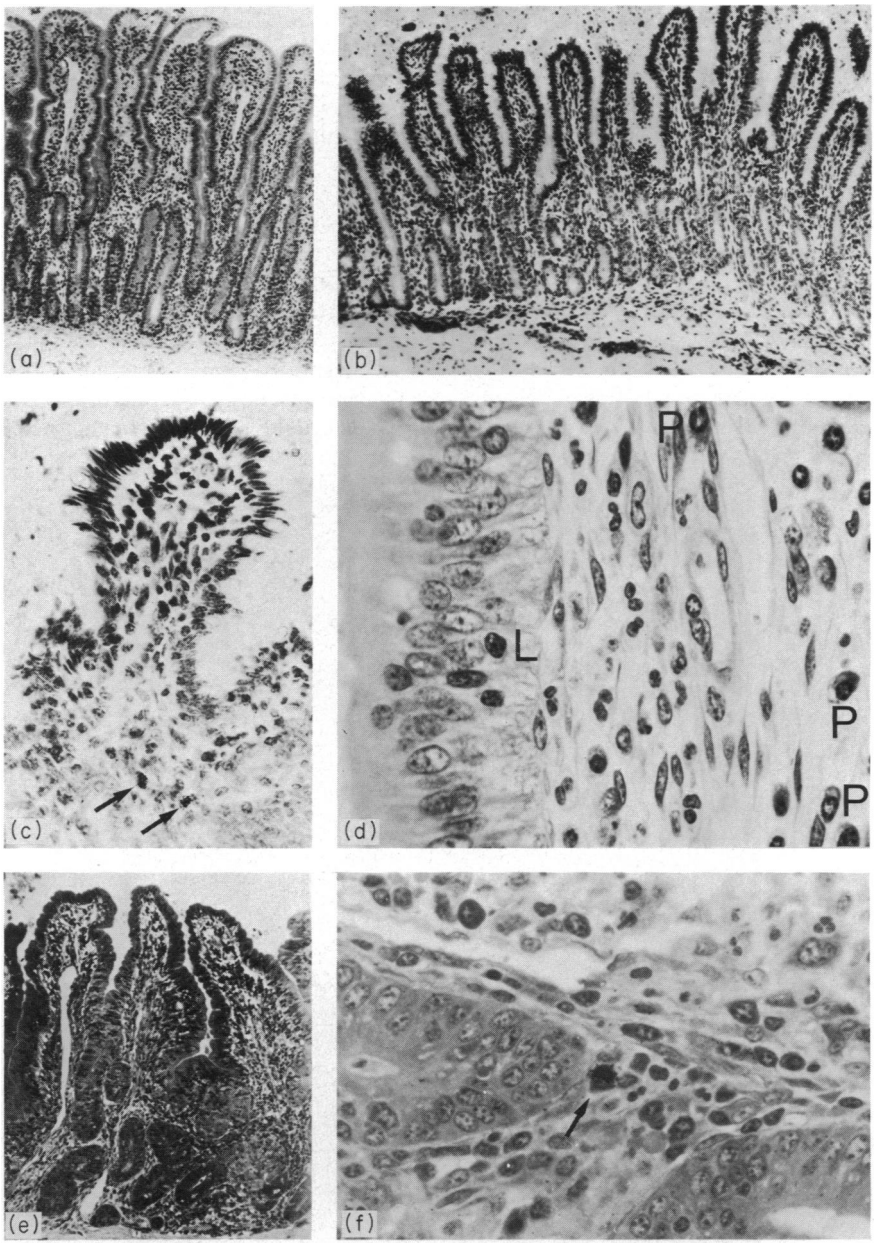

Figure 2. The postnatal development of the mucosa in a segment of ileum isolated from the functional ileum of a lamb at 128 days gestation (see Fig. 3). (a) At 1 month of age, the functional ileal mucosa contained many lymphoid cells (haematoxylin-eosin; magnification ×80). (b) The mucosa in the isolated ileal segment, also 1 month after birth, was much smaller and contained fewer lymphoid cells; part of the isolated ileal segment was reconnected into the functional ileum at this time (haematoxylin-eosin; magnification ×130) (c) An autoradiograph showing two labelled cells in the isolated ileal segment of the lamb at 4 months of age (Giemsa; magnification ×325). (d) The isolated ileal segment 4 months after birth; there are plasma cells (P) in the lamina propria and lymphocytes (L) in the mucosal epithelium (Nocht's azure-eosin; magnification ×455) (e) The mucosa at 4 months of age in the segment of isolated ileum that had been reconnected into the functional ileum 3 months earlier (haematoxylin-eosin; magnification ×80). (f) An autoradiograph of the functional ileal mucosa (same experiment as [c]), showing a labelled plasma cell within a group of plasma cells near the crypt of Lieberkühn (Nocht's azure-eosin; magnification ×455).

with material which probably originated from shed mucosal epithelial cells; the older animals contained more of this material than the younger ones. Biopsies were taken from the undistended part of the isolated ileal segments.

Development of the lamina propria

There was no difference in the appearance of the lamina propria of the functional ileum and that of the isolated ileal segment up until birth; both contained small lymphocytes but no lymphoblasts or plasma cells.

The lamina propria of the functional gut became transformed soon after birth; it increased rapidly in width and became infiltrated with many more lymphoid cells. By 10 days after birth, many plasma cells and lymphoblasts were present in the lamina propria. From 1 month of age, the lamina propria appeared to be fully developed (Fig. 2a). There were prominent clusters of plasma cells in the region near the crypts of Lieberkühn (Fig. 2f). This postnatal phase of rapid growth and maturation of the lamina propria failed to occur in lengths of ileum that had been isolated from the functional intestinal tract since before birth (Figs 2b, c, d). After birth, the villi in the isolated ileal segments remained small and the lamina propria acquired a sparse population of lymphoid cells (Figs 2b, c, d) together with some plasma cells and lymphoblasts (Fig. 2d). The development of the mucosa in the isolated ileal segments was defective for at least 2 years after birth.

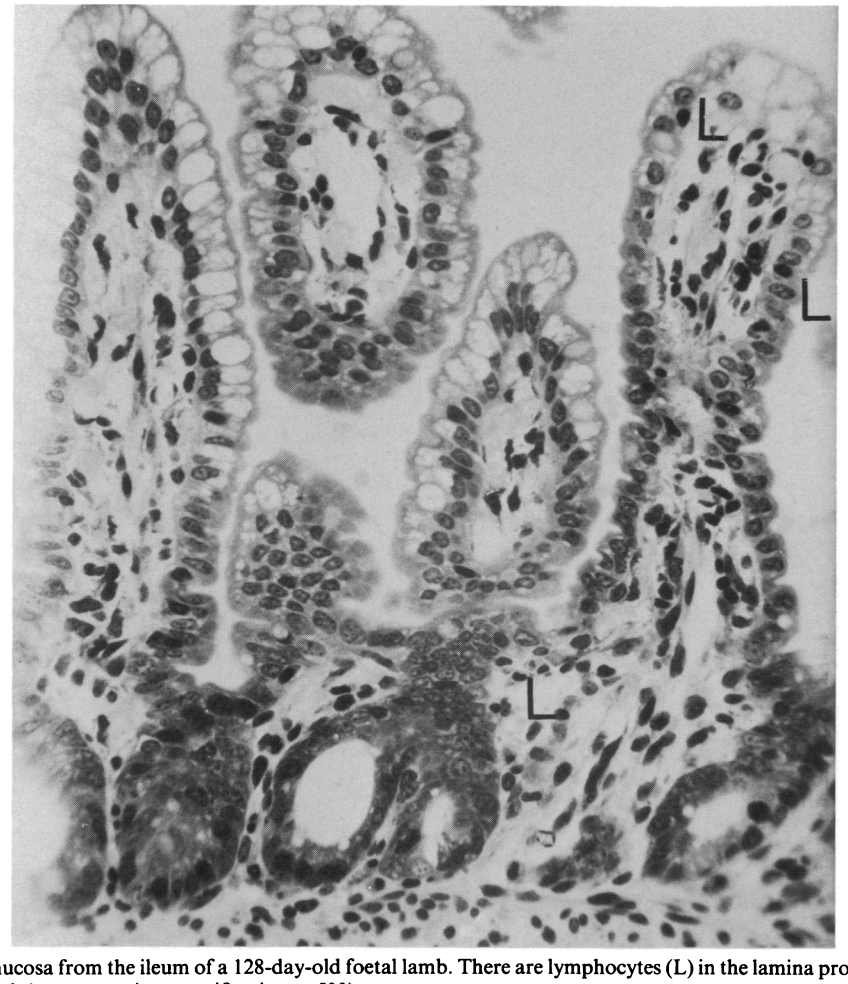

Figure 3. The mucosa from the ileum of a 128-day-old foetal lamb. There are lymphocytes (L) in the lamina propia and mucosal epithelium (Nocht's azure-eosin; magnification × 500).

Lymphoid cells in the intestinal epithelium

Lymphocytes were found in the epithelium of foetal lambs from as early as 50 days gestation (Fig. 3). An estimate of the frequency of these cells in the intestines of foetuses of different ages and in lambs after birth was made by counting the number of lymphocytes associated with 1000 epithelial cells in sections of the ileum where the epithelium had been cut vertically. These estimates showed that the frequency of intraepithelial lymphocytes increased gradually throughout gestation, and at birth there were 3–4 lymphocytes for every 100 epithelial cells (Fig. 4). The numbers of lymphocytes within the epithelium increased sharply after birth and reached about 30–35 lymphocytes for every 100 epithelial cells in lambs of 2 months of age (Fig. 4). This postnatal increase in the number of intraepithelial lymphocytes did not occur in the isolated ileal segments, where the frequency of these cells was about one sixth of that in the epithelium of the functional intestine of the same animal (Fig. 4).

Changes in the cell population in the mucosa of reconnected ileal segments

In six lambs, parts of the isolated ileal segments were reconnected surgically to the functional ileum between 1 and 4 months after birth (Fig. 1). Biopsies taken from the ileal segments, 3 months after they were reconnected into the gut, showed that the mucosa had expanded (Fig. 2e) and there were now normal numbers of intraepithelial lymphocytes (Fig. 4) and plasma cells.

The migration of lymphoblasts into segments of functional and isolated ileum

The intestinal lymph duct was cannulated in four lambs in which isolated segments of ileum had been previously established. The ages of these lambs varied from 1 to 8 months. The cells from an overnight lymph collection were separated, and incubated with [^{3}H]-thymidine to label the large blast cells and other cells undergoing DNA synthesis. The total cell population was reinjected into the lamb from which it had been collected, and the distribution of radioactivity in the various tissues was determined 40 hr later. The specific radioactivity of the mesenteric lymph nodes and the functional small intestines was two- to sevenfold greater then in the spleen and in those lymph nodes (prescapular and popliteal) remote from the gut

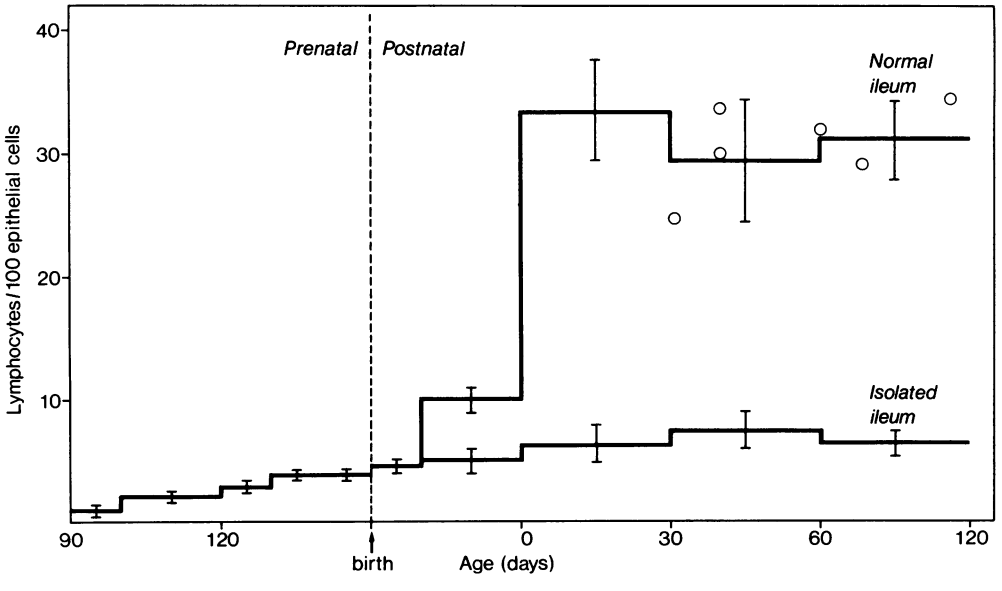

Figure 4. The number of lymphocytes per 100 epithelial cells in the intestinal epithelium of normal ileum, isolated ileum and ileum that had been reconnected into the intestinal tract after 1–3 months isolation. There are three to six animals in each age group (values are means ± standard errors) for normal and isolated ileum and one animal per point (O) for the reconnected ileum.

(Table 1). Autoradiography of the various tissues confirmed that the mesenteric lymph nodes and the intestinal lamina propria had the highest density of labelled cells, and many of them appeared to be plasma cells (Fig. 2f). Labelled cells were found in the isolated ileal segments (Fig. 2c), but the specific radioactivity of this tissues was on average only about 25% of the adjacent functional ileum (Table 1). The differences in the specific radioactivity of the isolated ileum and of the functional ileum following the injection of labelled cells appeared to be related to the length of time the segment had been isolated. In a one-month-old lamb, the specific radioactivity of the isolated segment was 50% of the functional ileum, in two lambs aged 3 months it was 20%, and in one lamb aged 8 months it was 10%. The specific radioactivities of the ileal segments that had been reconnected to the intestinal tract were similar to that of normal ileum (Table 1). These differences were confirmed by autoradiography.

DISCUSSION

The techniques used in the present experiments have allowed the examination of the effects that the gut digestive functions have on the development of the lymphoid cell populations in the ileal mucosa of lambs. This has been possible by the isolation of lengths of ileum from the functional gut of foetal sheep before they had had contact with foreign antigens at birth. After birth these isolated segments played no part in the normal digestion of food and they remained sterile.

Table 1. Distribution of radioactivity in various tissues of lambs with isolated and reconnected segments of ileum, 40 hr after an intravenous injection of cells from intestinal lymph which had been labelled *in vitro* with [^{3}H]-thymidine

Tissue	Radioactivity (d.p.m.) per g tissue	
Lung	3300 ± 1000	(6)
Spleen	9200 ± 2200	(6)
Popliteal nodes	5000 ± 1400	(6)
Mesenteric nodes	28,500 ± 7000	(6)
Jejunum	30,200 ± 6500	(6)
Ileum	17,200 ± 4200	(6)
Isolated ileum	4800 ± 1400	(6)
Reconnected ileum	16,300 ± 2300	(3)

The values are given as means ± standard errors: the number of animals in each group is shown in parentheses. The age of the lambs varied from 1 to 8 months. Three lambs (two aged 4 months and one 8 months) had had portions of previously isolated ileal segments reconnected into their intestinal tracts 1, 3 and 4 months earlier, respectively.

There were unequivocal differences between the number of lymphoid cells present in the mucosa of the isolated ileal segments and the number in the functional ileum. These differences disappeared when the isolated segments were reconnected into the gut so that they became involved in digestive functions. There seems little doubt therefore that the establishment and maintenance of the normal lymphoid cell population in the ileal mucosa depends on the participation of the gut in digestion and absorption.

There can be no simple explanation for the differences observed in the lymphoid development in the functional and the non-functional ileum. The attainment of a particular content and composition of lymphoid cells in a tissue such as the gut will certainly be the product of a complex set of interactions. The lymphoid tissues associated with the gut are subjected to a host of novel experiences after birth which may influence their subsequent development. The newborn lamb encounters foreign antigen for the first time, and the physiological activities associated with digestion and absorption initiate the secretion of enzymes and enhance the blood supply to the gut, which may in turn influence the rate at which lymphoid cells extravasate there (Hay & Hobbs, 1977). A study by Ottaway & Parrott (1980) showed in mice, as we have shown for sheep, that there is a greater traffic of lymphoblasts from the blood into the jejunum than into the ileum. These differences correlate well with the regional blood flow to the gut. It is therefore possible that the differences between the lymphoid content of the functional and the non-functional ileum may be partly related to the substantial differences in blood flow that would have existed between these tissues.

No doubt antigenic stimulation plays an important role in the establishment of the lymphoid cell populations in the gut mucosa. Plasma cells are never seen in the tissues of foetal lambs where antigen is not normally present, whereas they do appear when antigen is introduced into the intestinal lumen of the foetus (Reynolds, 1976). Also, the changes that occur in the intestinal lymph after birth, such as the increase in cell output and the appearance of lymphoblasts, is almost certainly an aspect of an immune response to foreign antigens absorbed from the gut (Cole, 1969; Reynolds, 1976).

Our observations and those of others (Hall, Parry & Smith, 1972; Husband & Gowans, 1978) have shown that lymph-borne blast cells are the progenitors of plasma cells in the gut mucosa. The experiments with the radiolabelled blast cells from intestinal lymph of lambs showed that fewer cells of this type accumulated in the isolated ileum than in the functional ileum. The low rate of accumulation of blast cells in the isolated ileum may be one reason for the paucity of plasma cells there. It is, however, difficult to define the physiological basis whereby the observed differences in cell numbers in the functional and non-functional ileum has occurred. The rate of migration of lymphoid cells from the blood into different tissues may vary considerably, and once cells enter a tissue, there are a variety of possible outcomes which may alter the numbers present at a particular time. For example, the extent to which cells are trapped and immobilized within a particular tissue may vary; they may be stimulated to proliferate or perish at different rates, or they may undergo differentiation (Morris, 1980). It may be that the presence of antigen induces lymphoblasts to accumulate in tissues and that this stimulus is lacking in isolated ileal segments. However, most investigators have failed to show any antigenic specificity in the migration of lymphoblasts (Rose, Parrott & Bruce, 1976; Hall, Hopkins & Reynolds, 1980). In fact, our experiments in sheep and other experiments in rats (Moore & Hall, 1972) show that lymphoblasts will migrate into the gut mucosa and mature into plasma cells even when the intestinal lumen contains no antigen.

The study which is most comparable with ours was that done by Parrott & Ferguson in mice. They grafted lengths of late-term foetal gut under the kidney capsule of syngeneic adult recipients, and found that there were fewer lymphoid cells in the lamina propria of these grafts than in the normal functional intestine (Ferguson & Parrott, 1972). They also reported that the number of lymphoblasts obtained from the mesenteric lymph nodes which localized in the intestinal grafts was not reduced (Parrott & Ferguson, 1974). The result of this experiment is thus quite different from ours.

The fact that no significant increase occurred postnatally in the number of intraepithelial lymphocytes in the isolated ileal segments suggested that digestive activity was also required to induce the normal postnatal development of this cell population. Ferguson & Parrott (1972) reached a similar conclusion from their studies in mice. As intraepithelial lymphocytes are present in the gut mucosa before birth, and as the kinetics of proliferation of intraepithelial lymphocytes cannot be interpreted simply as a response to antigens (Röpke & Everett, 1976), it seems that other factors unrelated to antigen are also important in the evolution of the lymphoid population in the mucosal epithelium.

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