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The response of gut-associated lymphoid tissue in gnotobiotic piglets to the presence of bacterial antigen in the alimentary tract

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INTRODUCTION

The alimentary tract of a piglet is bacteriologically sterile at birth, but within a few hours it is colonized by micro-organisms (Wilbur *et al.* 1960). The effect of this microbial flora on the histological structure of the intestine and its relation to the immune status of the piglet is largely unknown. The effect of intestinal organisms can, however, be studied in germ-free piglets. Such animals have not experienced antigens other than those in the diet and may be infected orally with a pure culture of a normal intestinal inhabitant such as *E. coli*.

In a previous study (Anderson, 1974) it was demonstrated that the palatine tonsil and costoaxillary lymph nodes of gnotobiotic piglets responded to oral administration of a live culture of a non-pathogenic *E. coli* by the formation of germinal centres. This response indicated an efferent pathway from the oral cavity to the tonsil and its regional lymph nodes. The presence of pyroninophilic cells in the prefemoral lymph node suggested an additional haematogenous dissemination of antigen-induced blast cells, though the original antigenic stimulus may well have occurred at a level of the alimentary tract other than the tonsil.

There is no lymphoid appendix in the pig; the gut-associated lymphoid tissue (GALT), besides that in the oral cavity, is concentrated in extensive Peyer's patches in the ileum and in scattered discrete nodules in the colon (Sloss, 1954). GALT can be identified in the fetus as aggregates of quiescent lymphocytes in the gut wall (Kruml *et al.* 1971; Chapman, Johnson & Cooper, 1974) and has a similar quiescent appearance in germ-free piglets (Kruml *et al.* 1969). This paper records the changes induced in the duodenum, ileum, mesenteric lymph node and prefemoral lymph node of gnotobiotic piglets following feeding with a culture of a non-pathogenic *E. coli*.

MATERIALS AND METHODS

Piglets. Three litters of germ-free piglets were obtained and maintained as previously described (Anderson, 1974). The gnotobiotic status of the piglets was determined by subculture of rectal swabs under aerobic and anaerobic conditions. Swabs were taken at weekly intervals, and from each piglet before killing.

Bacterial antigen. A strain of *Escherichia coli* which was non-enterotoxic and lacked the K88 antigen was grown overnight on glucose nutrient agar (Cruickshank, Duguid & Swain, 1965). The organisms were suspended in saline (0·15 M-NaCl), washed three times, and finally suspended in saline to yield approximately 10^{10} colony forming units per ml.

Experimental design. Each litter consisted of six piglets. When 7 days old each of the piglets in two of the litters received 15 ml of the suspension of *E. coli* in their milk (200 ml). Food was withheld for about 1 hour beyond normal feeding time to ensure that all the milk was taken. A pure culture of *E. coli* was obtained from rectal swabs 48 hours after dosing and thereafter from each piglet killed. The third litter acted as a control; the piglets received 15 ml sterile saline in their milk and organisms were not cultured from the rectal swabs at any time during the experiment.

One piglet from each litter was killed 1, 3, 5, 7, 9 and 11 days after exposure to the antigen or to the control suspending fluid. In this way there were two infected piglets and one uninfected piglet at each day of examination. At post-mortem specimens of the following tissues were removed for histological examination: one from the duodenum, approximately 15 cm from the pylorus; one from the ileum, approximately 15 cm from the ileo-caeco-colic valve; two from the mesenteric lymph node (one from the region draining the ileum and one from the region draining the duodenum) and one from the left prefemoral lymph node. The specimens were fixed in Carnoy, embedded in paraffin wax, sectioned at 5 μm, and stained with methyl green–pyronin or with haematoxylin and eosin. In addition, specimens of ileum were fixed in 12% neutral buffered formalin and stained with Giemsa's stain.

RESULTS

Uninfected piglets

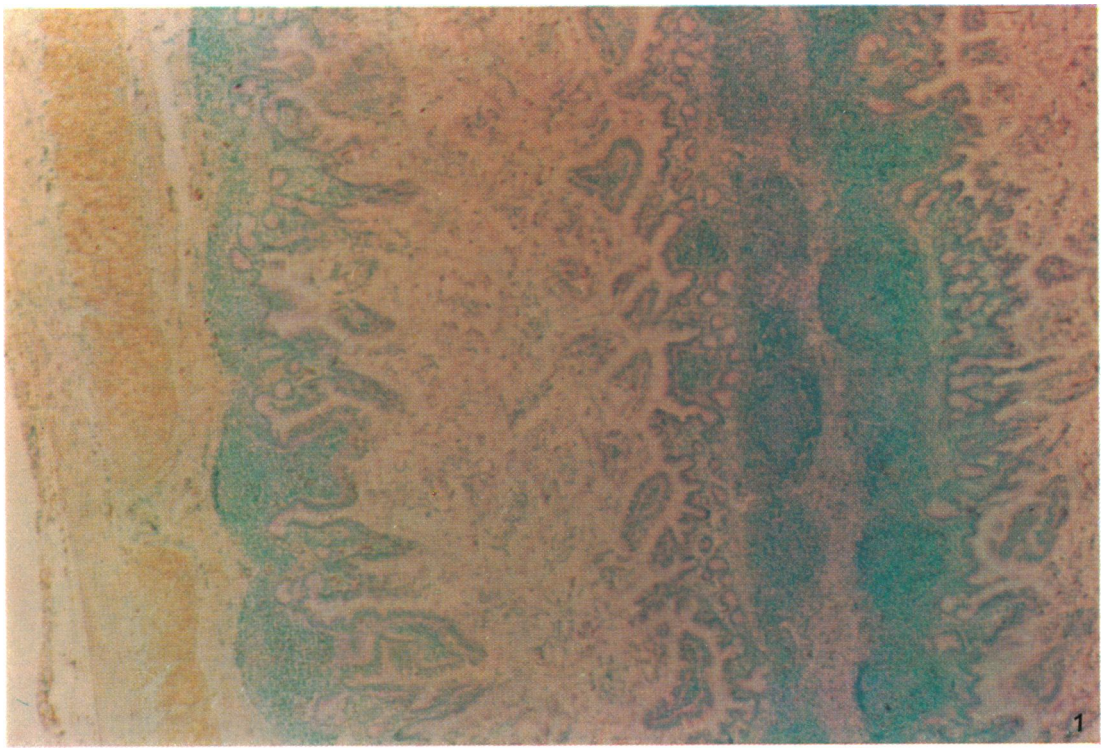
The histological features of the tissues of piglets killed at different times in the course of the experiment were similar, and are described together.

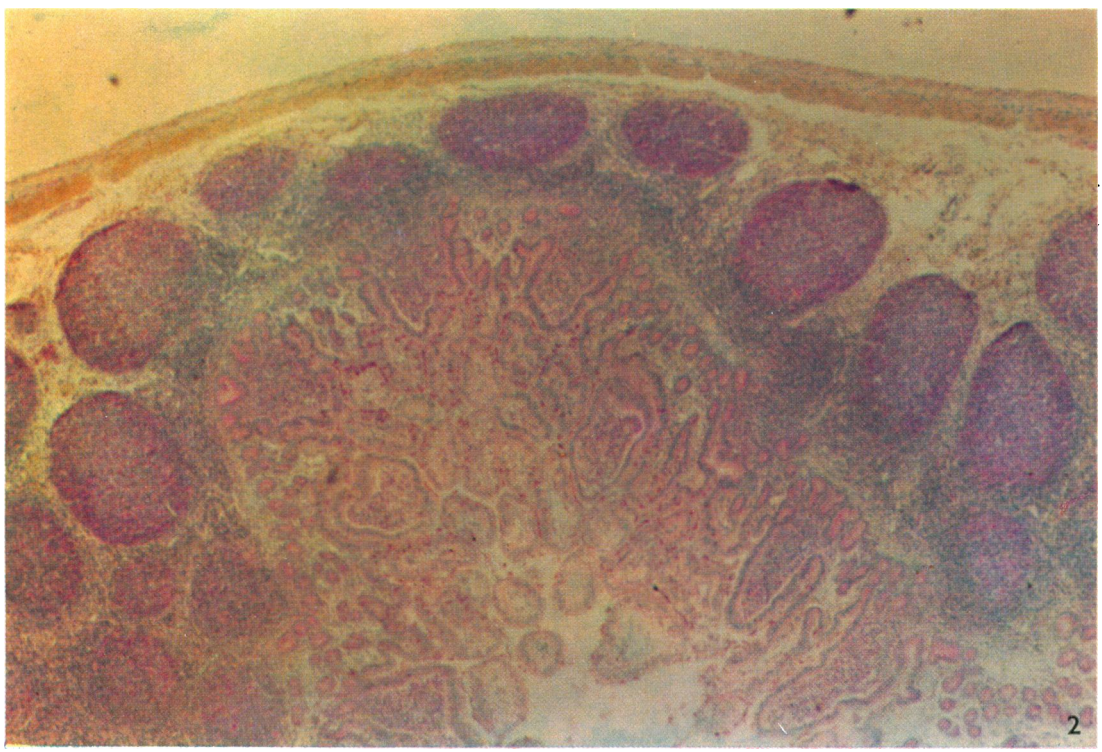
Duodenum

The distinguishing feature was the absence of cells of the plasma cell series from the lamina propria. In other respects the duodenum was normal in appearance.

Ileum

The villi were long and the epithelial cells were vacuolated, especially at the tip of the villi. There was little axial stroma and the lamina propria was sparsely populated with cells; eosinophils were present but there were no plasma cells. There were clumps or aggregates of lymphiod cells, mainly lymphocytes, in the submucosa. Generally only one such aggregate intervened between the muscularis mucosa and the muscularis externa, and a series of aggregates extended along the ileum (Fig. 1). In some areas the continuity of the muscularis mucosa was broken and the cells comprising the lymphoid aggregate extended through the muscularis into the lamina propria of the mucosa. The villus in such an area was low and formed a dome with pseudo-stratified columnar epithelium that was devoid of goblet cells. Lymphocytes were occasionally seen in the epithelial cells between the nucleus and the basement membrane, but the majority of lymphocytes were found in the richly vascularized lamina propria. The numbers of pyroninophilic blast cells in the lymphoid aggregates varied. There were none in most aggregates, but in some several pyroninophilic cells were present, and where a dome was sectioned pyroninophilic cells were occasionally seen amongst the cells under it. The triangle formed by adjoining aggregates and the epithelium – the thymus dependent area – was relatively deficient in lymphocytes,





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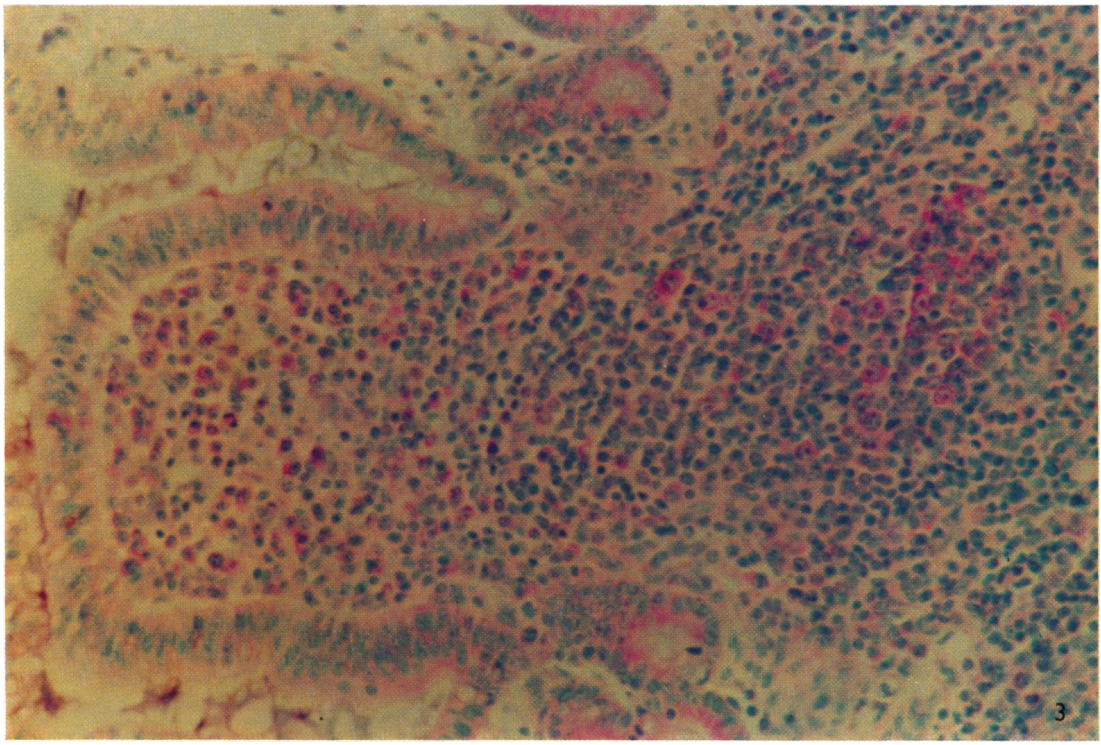

Fig. 1. Ileum of germ-free piglet 14 days old. The lymphoid aggregates in the submucosa, under the muscularis mucosa, lack pyroninophilic cells. MGP × 60.

Fig. 2 Ileum of gnotobiotic piglet 7 days after oral infection with live *E. coli*. There are numerous follicles in the submucosa which are rich in pyroninophilic cells. MGP × 60.

Fig. 3. High power of Peyer's patch showing the dome containing mixed cell types under the epithelium, the lymphocyte-rich neck and the follicle which consists of pyroninophilic cells. MGP × 585.

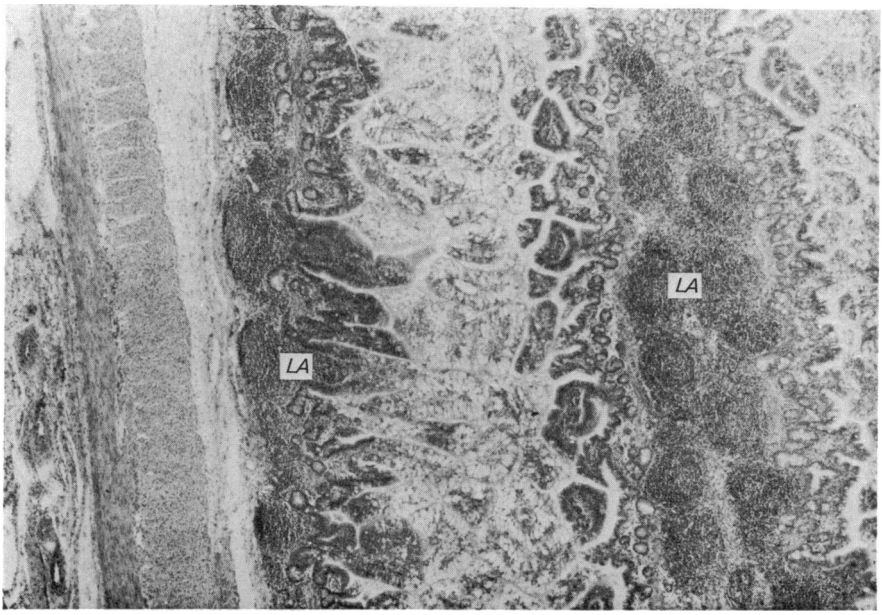

Fig. 1. Ileum of germ-free piglet 14 days old. There are lymphocyte aggregates (*LA*) in the submucosa. H & E. × 60.

and the triangle formed by adjoining aggregates and the muscularis externa contained blood vessels and lymphatics.

Mesenteric and prefemoral lymph nodes

The histological appearance of the mesenteric lymph node and of the prefemoral lymph node was similar to that of other lymph nodes from germ-free piglets (Anderson, 1972). There was a small area of quiescent cortex in which a few pyroninophilic cells were found, but there were no germinal centres. The medulla consisted of a histio-reticular network in which venules were conspicuous owing to the paucity of lymphocytes.

Infected piglets

Duodenum

The lamina propria of the duodenum of infected piglets remained devoid of cells of the plasma cell series until 7 days after infection, when pyroninophilic blast cells and immature plasma cells were seen. These cells were present 9 and 11 days after infection, and occasionally mature plasma cells were observed.

Ileum

The long vacuolated villi characteristic of the germ-free state were absent 3 days after infection.

The number of pyroninophilic cells in the submucosal lymphoid aggregates was obviously greater 1 day after infection than in the uninfected piglets, and there were mitotic figures. On subsequent days the number of pyroninophilic cells in the submucosal aggregates rapidly increased, with the majority accumulating at the base and towards the sides of the aggregates to form follicles (Fig. 2). Where a dome was sectioned longitudinally there was an area of small lymphocytes at the level of the

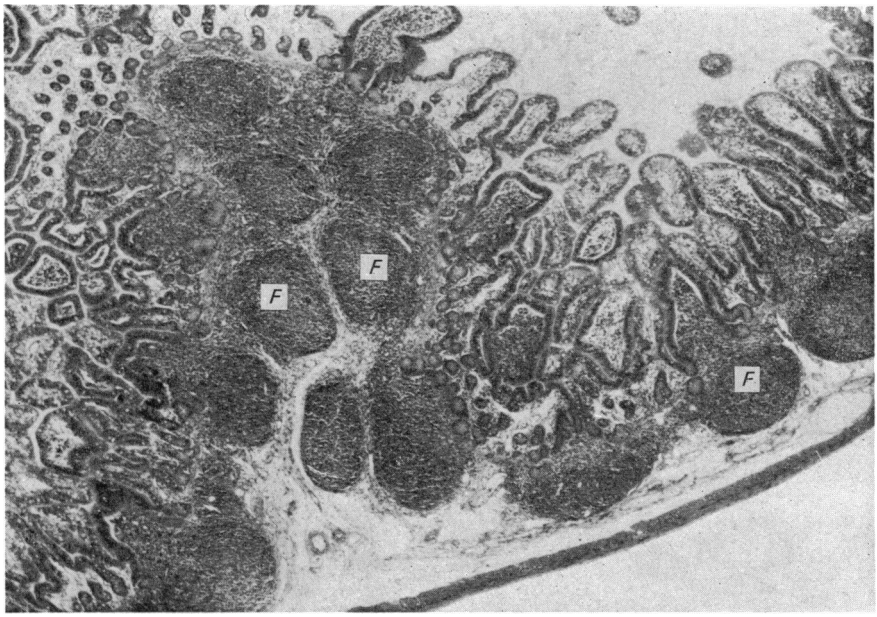

Fig. 2. Ileum of gnotobiotic piglet 7 days after oral infection with live *E. coli*. There are numerous follicles (*F*) in the submucosa. H. & E. × 60.

muscularis mucosa where small venules were visible, and under the dome there was a mixed cell area that contained pyroninophilic blast cells and lymphocytes (Fig. 3). Some pyroninophilic cells had a leptochromatic nucleus and lacked a nucleolus and were interpreted as an early stage in the transformation of lymphocytes. These transitional cells were seen in the dome epithelium as well as in the lamina propria under the dome. In sections in which domes were cut transversely their mixed cell core distinguished them from ileal villi.

There was no cellular expansion of the thymus dependent area and there were no mature plasma cells in the lamina propria or submucosa. Eosinophils were present in the lamina propria throughout the period of observation, but were never seen in a lymphoid aggregate or in a dome. Neutrophils appeared in blood vessels and adjacent tissue at the edge of aggregates 1 day after infection, and subsequently they were found in the lamina propria, but this response was transient.

Mesenteric lymph node

There was no difference between the lymph node of uninfected piglets and those of infected piglets 1 day after oral infection, and thereafter there was a similar response in both samples of the mesenteric lymph node of infected piglets. Three days after infection there were pyroninophilic cells in the subtrabecular sinuses and some were randomly scattered in the cortex. There were more of such cells in the subtrabecular sinuses and in the cortex 5 days after infection, and aggregates of pyroninophilic cells, i.e. early germinal centres, were found in the cortex at 7 days. Small lymphocytes were identified in the medullary sinuses adjacent to the cortex. Nine and 11 days after infection numerous germinal centres were present in the cortex, but there were no plasma cells in the medulla.

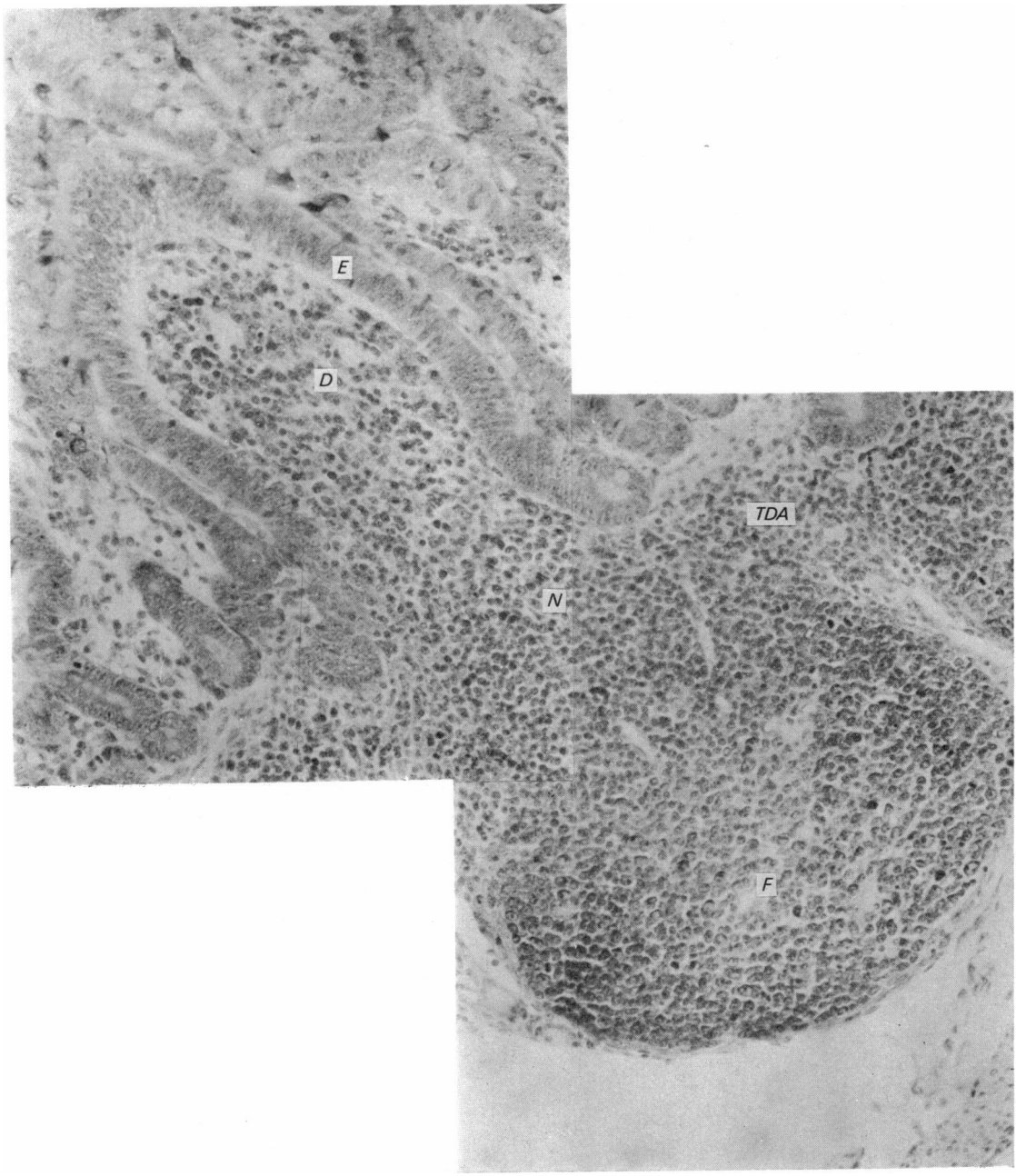

Fig. 3. High power of Peyer's patch showing the dome (*D*) containing mixed cell types under the epithelium (*E*), the lymphocyte-rich neck (*N*) and the follicle (*F*) which consists of pyroninophilic cells. The thymus-dependent area (TDA) is identified. MGP. × 585.

Prefemoral lymph node

The lymph nodes examined 1, 3 and 5 days after infection were similar to lymph nodes from un-inoculated piglets. Seven days after infection there were pyroninophilic cells in the subtrabecular sinuses. Thereafter pyroninophilic cells were also found scattered in the cortex, but they were not aggregated into early germinal centres and there were no plasma cells in the medulla.

DISCUSSION

The outstanding observation in this study was the rapid and intense proliferation of cells in the lymphoid aggregates of the ileum in response to the introduction of bacteria, with the formation of follicles. Thus, without recourse to surgical interference or X-irradiation, the necessity of intestinal bacteria for the development of normal GALT architecture was demonstrated.

At 50 days of gestation in the pig the lymphoid cells of the gut are confined to the lamina propria below the dome and above the muscularis mucosa and only at 90–100 days of gestation are submucosal aggregates present (Chapman *et al.* 1974). The migration route of the lymphoid cells appears to be from the dome to the submucosa. In this study similar aggregates of lymphoid cells in the mucosa and submucosa which were almost devoid of pyroninophilic blast forms were seen in uninfected germ-free piglets. Lymphocytes were occasionally detected within the dome epithelium, and the underlying lamina propria was rich in blood vessels in which there were lymphocytes. These observations support the close association between lymphocytes and epithelium, in a relatively antigen-free situation, which appears to be fundamental to primary lymphoid tissue (Fichtelius, 1967; Edwards, Murphy & Cho, 1975).

The epithelial cells of the dome specialize in pinocytosis and are probably responsible for the transport of antigenic material across the epithelium into the lamina propria of the dome (Bockman & Cooper, 1973; Owen & Jones, 1974). Results in this paper suggest that antigen stimulates lymphocytes (B-lymphocytes) to transform to pyroninophilic cells in the lamina propria of the dome or in the dome epithelium, and that these cells migrate to, and accumulate in, the submucosa, forming a follicle in which proliferation occurs. This sequence of events has been suggested by Waksman, Ozer & Blythman, (1973) as occurring in the rabbit appendix as a non-specific B-cell amplification mechanism, stimulated by bacterial endotoxin. The technique employed in the present study could not distinguish between an antigenic and a mitogenic mechanism; it is likely that both mechanisms operate.

Soon after infection blast cells were evenly distributed within follicles, but by 11 days division into a cortex and medulla was apparent, especially in those follicles that were cut transversely below their domes. The cortex–medulla organization, which is not assocated with separation by a basement membrane as in the follicles of the bursa of Fabricius, may simply reflect the vasculature. Drainage by the lymphatics and veins in the centre of the follicle would result in a thinning of the cell population in that area with consequent concentration of actively dividing cells at the periphery.

The formation of germinal centres in the mesenteric lymph node indicated drainage of antigen and antigen-induced pyroninophilic cells to the lymph node from GALT, and this is consistent with known lymphatic pathways (Sisson, 1953). The presence

of pyroninophilic cells, but no germinal centres, in the prefemoral lymph node suggested a haematogenous spread of cells following antigenic stimulation of lymphoid tissue in the alimentary tract, although in this respect the relative contribution of tonsils and Peyer's patches is unknown. Neither lymphocytes nor plasma cells were found in the lamina propria of the duodenum of uninfected piglets or in infected piglets before 7 days after infection, but at this time, and thereafter, pyroninophilic cells were observed, and this was probably also a result of the haematogenous spread of GALT stimulated cells. In the lamina propria of the duodenum pyroninophilic cells apparently transformed into plasma cells, but plasma cells were not seen in either the mesenteric or prefemoral lymph nodes.

Attempts to identify immunoglobulin-producing cells in pig tissues by staining with fluorescein isothiocyanate conjugated anti-pig immunoglobulins (Atkins, Schofield & Reeders, 1971; Allen & Porter, 1973) have been complicated by both primary (auto-) fluorescence and non-specific secondary fluorescence of eosinophils (Brown, Bourne & Steel, 1974). However, histological studies (Anderson, 1973) and studies using horseradish peroxidase conjugated anti-pig immunoglobulins (Brown & Bourne, 1976) indicate that antibody production is not a major function of lymph nodes in this species. On the other hand, the lamina propria of the gut appears to provide a suitable micro-environment for plasma cell proliferation, and it is suggested that in the pig the immunoglobulin-producing cells of the respiratory and alimentary mucosae provide circulating IgG, IgM and IgA as well as the immunoglobulins of external secretions (Bradley, Bourne & Brown 1976; Brown & Bourne, 1976).

A field trial, in which the live weight gains of piglets orally immunized by continuous feeding of *E. coli* antigens were compared with the live weight gains of piglets not fed the dietary additive, showed a significant weight gain in antigen fed piglets (Porter *et al.* 1973). This was attributed to an increased resistance to bacterial challenge which was mediated by the induction of local antibody production in the alimentary tract (Porter *et al.* 1974). The present study suggests that such an oral antigen would act primarily on the lymphoid tissue of the ileal wall, and secondarily on the mesenteric lymph node. In addition, pyroninophilic cells stimulated in GALT would enter the circulation, extravasate into the lamina propria of the upper alimentary tract, and develop to plasma cells.

SUMMARY

The cellular changes in the ileum and duodenum and in the mesenteric and prefemoral lymph nodes of gnotobiotic piglets were observed following feeding with a live culture of a non-pathogenic *E. coli*. There was a rapid and intense reaction in the lower ileum, and follicles were formed; germinal centres were formed in the mesenteric lymph node after a short delay. Germinal centres were not seen in the prefemoral lymph node, though there were pyroninophilic cells in the cortex of this node. Plasma cells were not detected in the medulla of either the mesenteric or the prefemoral lymph nodes, but pyroninophilic cells and plasma cells were found in the lamina propria of the duodenum from 7 days after infection onwards. These observations demonstrate the requirement of an intestinal flora for the development of normal Peyer's patch architecture and indicate that the Peyer's patch response secondarily affects the mesenteric lymph node. The observations also suggest that there is a haematogenous dissemination of pyroninophilic cells from gut-associated

lymphoid tissue to, amongst other sites, the duodenum; this may be of importance in both natural and artificial immunization by the oral route.

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