Cryptosporidiosis and the follicle-associated epithelium over the ileal Peyer's patch in calves

T. LANDSVERK, *Department of Pathology, Norwegian College of Veterinary Medicine, PO Box 8146, Dep, 0033 Oslo 1, Norway*

Three calves were studied in stages of spontaneous cryptosporidial infection with particular reference to the relation of the cryptosporidia to the follicle-associated epithelium (FAE) over the ileal Peyer's patch (IPP). In early infection scanning electron microscopy and streptavidin immunoperoxidase staining showed marked predilection of cryptosporidia for the FAE. Cryptosporidial antigen was also found in subepithelial tissue, both in the domes over the IPP and in villi, apparently in macrophages, where the parasites seemed to be progressively degraded. The FAE showed long tightly spaced microvilli, replacing normal low folds and protrusions, particularly in late infection. Endocytosis of indian ink was restricted to the cell periphery in late infection, contrasting the normal, more even distribution of endocytosis in the FAE apical cytoplasm. Few parasites were seen in the intestinal mucosa at this stage. At convalescence the FAE was normal, but all stages of infection were characterised by elongation of microvilli in absorptive cells.

IN a previous study frequent lesions of the follicle-associated epithelium (FAE) over the ileal Peyer's patch (IPP) were associated with spontaneous diarrhoea in calves where rotavirus and chlamydia seemed to be the major pathogenic agents (Landsverk 1981b). The present study concerns another frequent infection in calves, cryptosporidiosis (review, Angus 1983) with respect to changes in the FAE. Such studies have attracted particular interest after the demonstration that the IPP is responsible for the antigen-independent primary generation of B-lymphocytes in sheep (Reynolds and Morris 1984, Gerber et al 1986). Disturbances of the IPP function arising as a result of infections may thus have consequences for the individual's ability to develop immunity later in life. Since changes of the FAE often may be of an apparently persistent nature (Landsverk 1981b) emphasis is given to examination of both early and late phases of the disease. Some attention is also given to the possibility that the FAE may function in antigen transfer to immunocompetent lymphoid tissue.

Materials and methods

Three Norwegian Red calves were examined (calves 1, 2 and 3), two males and one female, aged 25, 20 and 30 days, respectively, on the day of killing. The calves had been raised conventionally, with colostrum immediately after birth and thereafter whole cow's milk, and later a milk replacer. Spontaneous diarrhoea occurred in all three calves. They were kept in neighbouring pens. Calf 1 was killed at the start of the diarrhoea, calf 2 in the late phase of the diarrhoea (11 days after the start of diarrhoea) and calf 3 eight days after the cessation of diarrhoea, which, for this calf, lasted for 10 days. Faeces in calves 2 and 3 were mucinous, whitish yellow and sometimes contained small amounts of blood.

In the acute stage of the disease cryptosporidia were found in faecal smears stained with modified Ziehl-Neelsen stain (Henriksen and Pohlenz 1981) or the streptavidin-biotin immunoperoxidase technique (see below). Examination of faeces for other pathogenic parasites, bacteria and viruses was negative. The methods used in the bacteriological examinations have been described (Landsverk et al 1983). Direct electron microscopy of faeces was done on faecal suspensions prepared as described by Flewett (1978), placed on carbon-Formvar coated grids, dried and negatively stained in potassium phosphotungstate. Frozen sections examined for rotavirus and corona-virus were likewise negative; the methods for immunofluorescence have been described (Landsverk et al 1983). Three healthy calves, 18, 20 and 26 days old, two females and one male, from the same farm, were used as controls.

Intestinal specimens were obtained through a right flank laparotomy during anaesthesia with pento-barbital (20 mg kg⁻¹ bodyweight). Before sampling, endocytic ability of the FAE over the IPP was tested by injecting a solution of 5 to 10 per cent colloidal carbon (Pelican C11/1431 A) in saline or phosphate buffered saline into a 10 cm tied loop at the ileal insertion of the ileocaecal fold. Various sites in the small and large intestine were sampled, including

proximal and distal portions of the IPP. After 30 minutes the loops were removed and immersed in a fixative containing 0·7 per cent paraformaldehyde, 0·9 per cent glutaraldehyde, and 140 mmol litre^{-1} sodium cacodylate, pH 7·2. Specimens for morphometry were fixed in Carnoy's solution.

An adjacent portion of the IPP was frozen in monochlorodifluoromethane (Prestogas) chilled with liquid nitrogen. Tissue was stored at −70°C until tested. Cryostat sections about 8 μm were fixed in acetone for 10 minutes. Alternatively, sections were fixed in 4 per cent formaldehyde, 67 mmol litre^{-1} calcium chloride, pH 7·2 for five minutes and then treated with 3 per cent hydrogen peroxide in methanol for 30 minutes to block the endogenous peroxidase. Immunoperoxidase staining for cryptosporidia included human con-

valescent serum as a source of primary antibody. Normal human serum was used as a control. The sera were obtained from Dr W. L. Current, Eli Lilly, Indianapolis and Dr R. Bergquist, Ulleval Hospital, Oslo. Biotinylated sheep anti-human Ig (Amersham, RPN 1003) was used as a secondary antibody. Streptavidin-biotinylated horseradish peroxidase complex (SBC) (Amersham, RPN 1051) was then added. Finally the sections were treated with a diaminobenzidine (DAB) hydrochloric acid solution (Sigma) containing 6 mg 10 ml^{-1} DAB, 45 μl 3 per cent hydrogen peroxide 10 ml^{-1} of a 50 mmol litre^{-1} imidazole solution (Boehringer Mannheim), pH 7·4. Dilution of sera and washing between each of the steps in the procedure was made with phosphate buffered saline.

Pieces of fixed tissue for light microscopy (LM) were

FIG 1: Ileal Peyer's patch, control. The dome (d) contains lymphoid cells and is outlined by a follicle-associated epithelium devoid of goblet cells. The dome is connected with the follicle (f) and the interfollicular T cell area (t). H&E, × 180
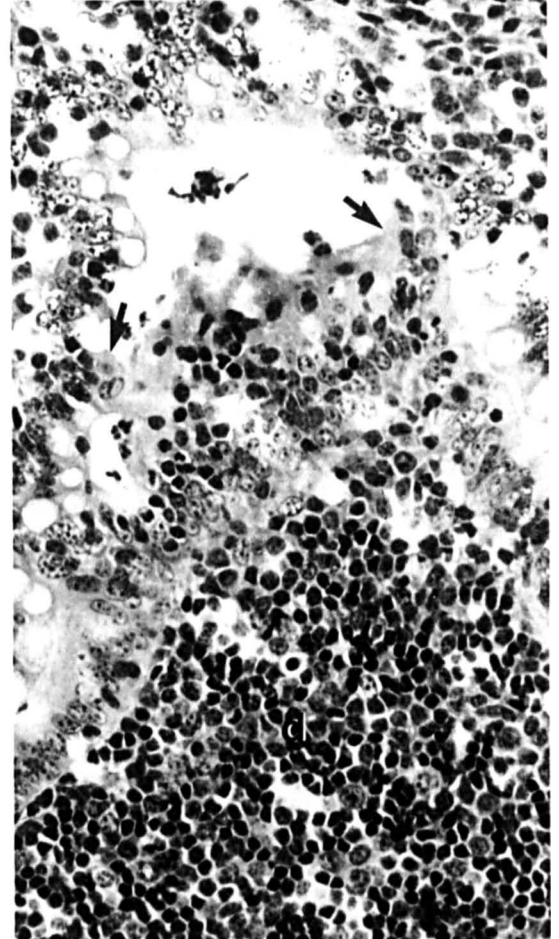

FIG 2: Ileal Peyer's patch, early cryptosporidial infection. The follicle-associated epithelium outlining the dome (d) shows fusion (arrows) with the adjacent absorbing epithelium. H&E, × 300
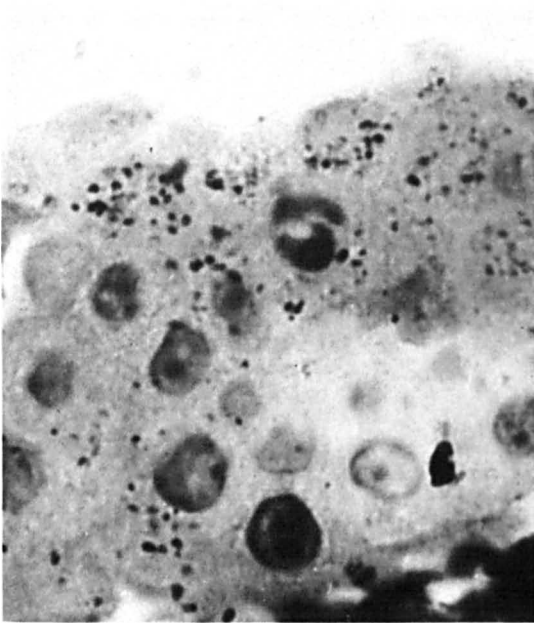
FIG 3: Ileal Peyer's patch, control, injection of colloidal carbon. Obliquely cut section showing normal distribution of endocytised carbon particles in the follicle-associated epithelial cells. H&E, × 1500
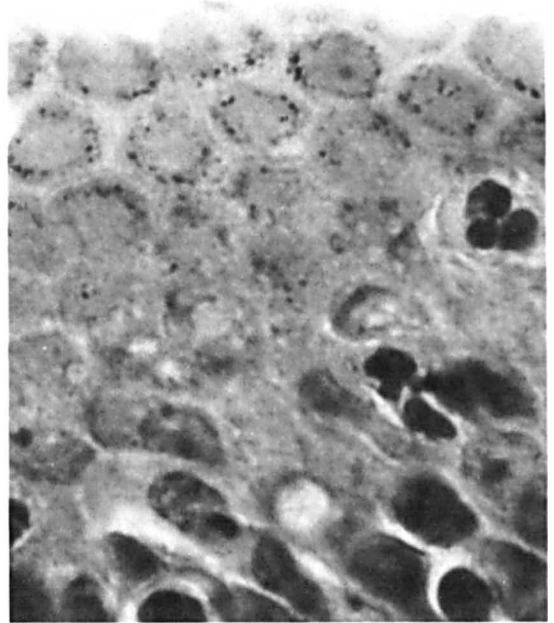

FIG 4: Ileal Peyer's patch, late cryptosporidial infection. Obliquely cut section showing concentration of endocytised carbon particles to the periphery of the follicle-associated epithelial cells. H&E, × 1500

dehydrated in ethanol, equilibrated in xylene and embedded in paraffin. About 5 μm thick sections were stained with haematoxylin and eosin (H&E), and modified Ziehl-Neelsen (Henriksen and Pohlenz 1981). Morphometric examination of follicle size was performed as described elsewhere (Landsverk 1981b).

Before processing of specimens for scanning electron microscopy (SEM) digital pressure fracturing (Miyai 1978) was performed on fixed tissue. The procedure frequently exposed the surface of the domes, otherwise often inaccessible with the conventional sectioning of tissue. The specimens were then washed in distilled water, dehydrated in acetone, critical-point dried, and sputter-coated with gold. The coated samples were examined in a Jeol 50 A scanning electron microscope (Jeol, Akishama, Tokyo), with an accelerating voltage of 10 to 15 kV. Photographs were recorded on a Polaroid type 52 film.

Specimens for transmission electron microscopy (TEM) were post fixed in 2 per cent cacodylate buffered osmium tetroxide for two hours, dehydrated in acetone and embedded in Epon. The ultrathin sections were cut with diamond knives and stained with uranyl acetate and lead citrate.

Results

LM revealed a heavy infection of cryptosporidia in calf 1 (early infection), affecting mainly the distal small intestine. Over the IPP cryptosporidia were more numerous in the FAE than in the adjacent absorbing epithelium. Villi showed partial atrophy and had a low irregular epithelium infiltrated with leucocytes. The crypts were elongated. Fusion of the FAE with absorbing epithelium was sometimes seen (Figs 1 and 2). Calf 2 (late infection) had only a few cryptosporidia in the brush border, whereas no cryptosporidia were seen in calf 3 (convalescent). Fusion of the FAE and absorbing epithelium was only rarely seen in calves 2 and 3.

Injection of colloidal carbon into intestinal loops showed an abnormal pattern of carbon particle distribution for calf 2. Whereas particles were seen evenly distributed throughout the apical cytoplasm of the FAE in the controls, calf 2 had particles concentrated to the periphery of the epithelial cells, giving ring-shaped staining in tangential sections (Figs 3 and 4).

Morphometric estimation of follicle size did not reveal any significant differences between calves infected with cryptosporidia and controls.

Immunoperoxidase staining with the SBC technique revealed numerous cryptosporidia in the luminal border of the FAE in calf 1 (Fig 5). Cryptosporidial antigen was also found subepithelially in the dome, apparently within macrophages. Cryptosporidia were less numerous in villi and usually associated with
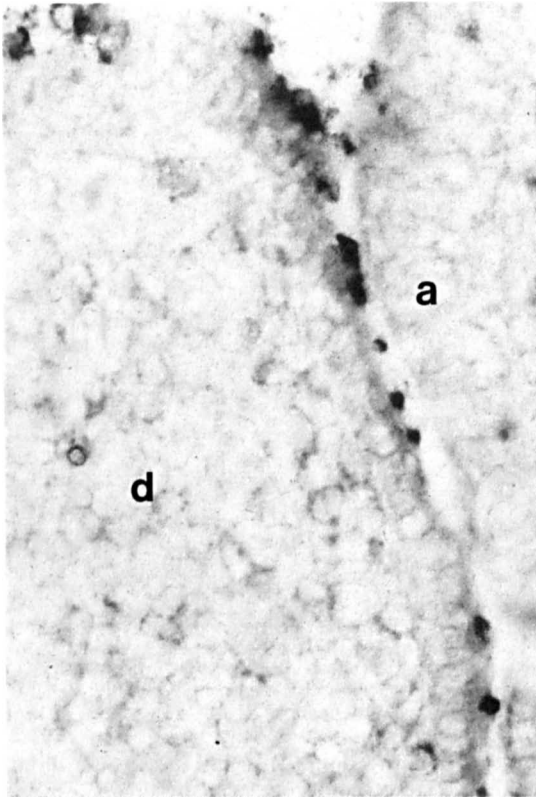
FIG 5: Ileal Peyer's patch, early cryptosporidial infection. Staining with antibodies against cryptosporidia, SBC technique. Many cryptosporidia are seen in the luminal membrane of the follicle-associated epithelial cells outlinine the dome (d) and few in absorbing cells (a). × 380
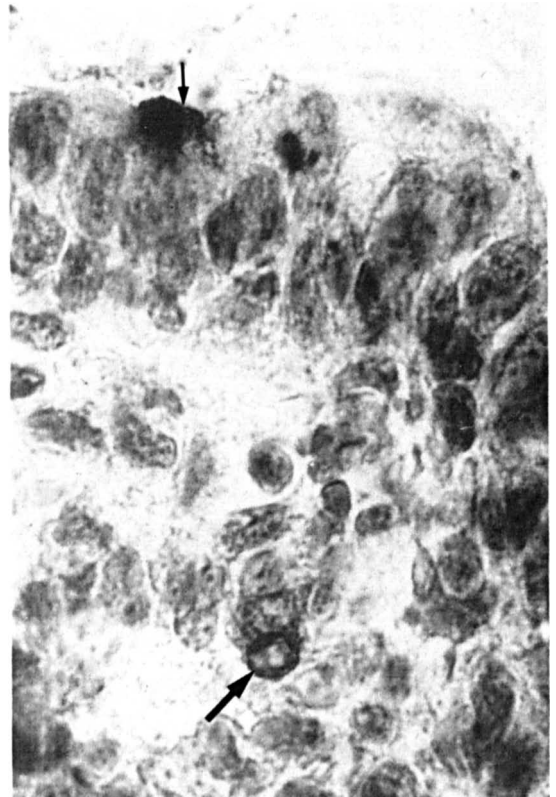
FIG 6: Distal jejunum, early cryptosporidial infection. Staining with antibodies against cryptosporidia, SBC-technique. A cryptosporidium within the epithelial brush border is indicated (small arrow), and cryptosporidial antigen within a probable macrophage (large arrow). Counterstained with haematoxylin, × 1200

villous tips. They were also smaller, probably being composed of younger stages. In formol-calcium fixed sections, where the endogenous peroxidase had been inhibited by hydrogen peroxide treatment, cryptosporidial antigen was seen in apparent macrophages in the lamina propria of the villi over the IPP as well as proximal to the IPP (Fig 6). Sometimes the reacting material within the leucocytes had the appearance of the brush border cryptosporidia. More often, smaller particles were found, probably formed by degradation of larger particles. In the apparent final stages of degradation a faint granular staining was seen within the leucocytes. Faecal smears taken in the acute phase of diarrhoea and stained with the SBC technique or the modified Ziehl-Neelsen stain verified the presence of cryptosporidia at this stage also in calves 2 and 3.

SEM confirmed the presence of cryptosporidia in calf 1. More parasites were associated with the FAE than with the absorbing epithelium (Fig 7). The organisms varied in size, apparently corresponding to developmental stages. The characteristic concentric ridges seen in the luminal membrane of the FAE in normal calves at this age (Fig 8) were mostly apparent, although often somewhat irregular (Fig 9). In addition, many cells had a flattened surface without ridges or microvilli, interpreted as remodelled epithelial cells closing epithelial defects (Fig 7). Ordinary villi were shortened, had an uneven surface, and microvilli of varying length.

In calf 2 occasional oocyst-like structures were seen at the mucosal surface; endogenous stages were rare. The FAE showed marked disappearance of surface ridges, the surface containing a central tuft of elongated tightly spaced microvilli and a peripheral flattened zone. Calf 3 had a normal FAE, and no parasites were found. Both calves 2 and 3 showed marked irregularities in the microvillous zone of the absorptive epithelium in the ileum and the colon, having the appearance of an unevenly cut lawn (Fig 10). The apparent variation in microvillous
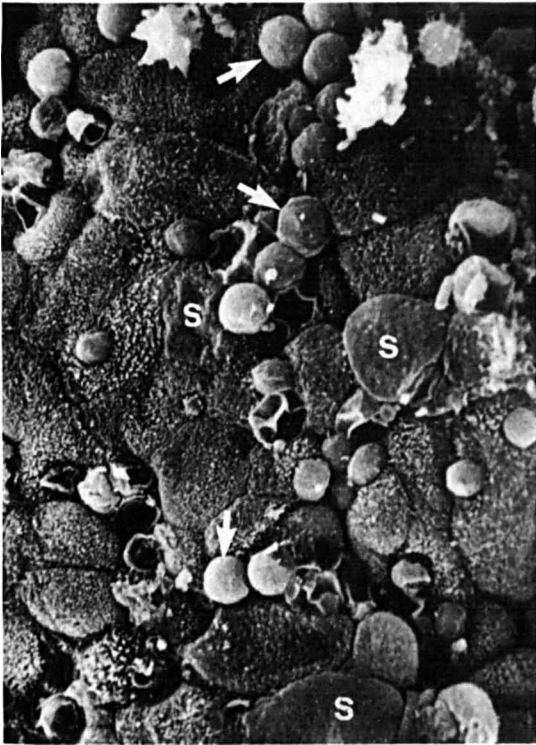
FIG 7: Scanning electron micrograph, ileal Peyer's patch, early cryptosporidial infection. Numerous cryptosporidia of different developmental stages (arrows) are inserted into the luminal membrane of the follicle-associated epithelial cells. The follicle-associated epithelial cells have short sparse microvilli, few folds or sometimes a smooth surface (s). ×2000
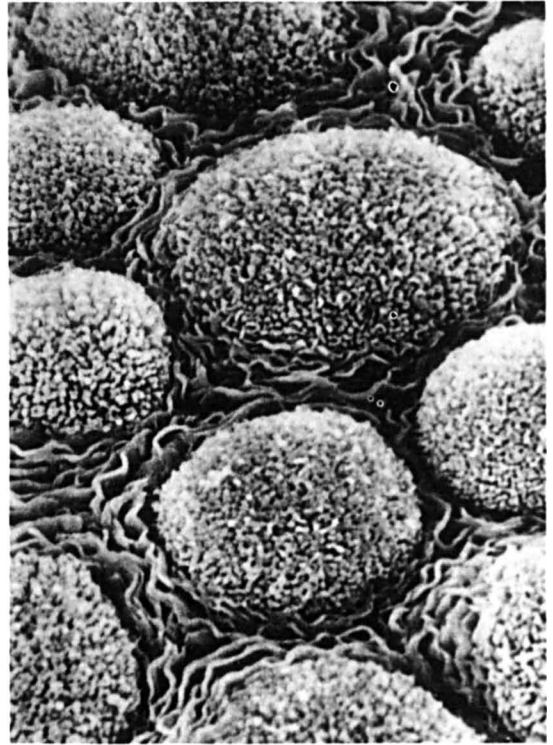
FIG 8: Scanning electron micrograph, ileal Peyer's patch, control. Follicle-associated epithelial cells with characteristic concentric folds encircling a central area with microvilli. ×5000

length between adjacent populations of absorbing epithelial cells was not seen in any of the controls.

TEM showed the various stages of parasite development in calf 1, including trophozoites, gametocytes and schizonts. The parasites in the FAE were often found in stages of degeneration. Macrophages within and beneath the FAE often contained cryptosporidia-like structures within phagosomes, although the structures could not be convincingly identified because of the lysosomal degradation. TEM confirmed the SEM changes of the FAE luminal membrane of calves 1 and 2. Microvilli tended to be longer than normal, particularly in calf 2. The elongated microvilli contained longitudinal filaments inserted deeply into the apical FAE cytoplasm (Figs 11 and 12). In the central microvillous zone of FAE of calf 2 vesicles interpreted as endocytic were present, although rare. Most of the endocytosis, as judged from the uptake of colloidal carbon, appeared to take place in the periphery of luminal membrane of this calf. Cryptosporidia were rare in calf 2. Calf 3 had a normal FAE morphology and no parasites were seen.

Discussion

The present study indicates a marked predilection of cryptosporidia for the FAE over the IPP. Previously, the same has been suggested for chlamydia (Landsverk 1981b) and astrovirus (Wood et al 1984). There are studies in other mammalian species suggesting that the PP is also susceptible to infections (Sprinz et al 1956, La Brec and Formal 1961, Carter and Collins 1974, Hohmann et al 1978), although the ruminant FAE over the IPP is of a type different from that so far described over the PP in other mammals. The properties of FAE over the IPP that may contribute to a predilection by microorganisms are not known. Among the possible factors may be absence of goblet cells in the FAE, a modified cell surface and enzyme activity (Torres-Medina 1981, Landsverk 1981a) and a low frequency of antibody secreting plasma cells in the domes (T. Landsverk, unpublished). Of interest is the recent demonstration that the FAE over the human PP is deficient in secretory component, which suggests that this FAE lacks IgA transporting capacity (Brandtzaeg 1985).
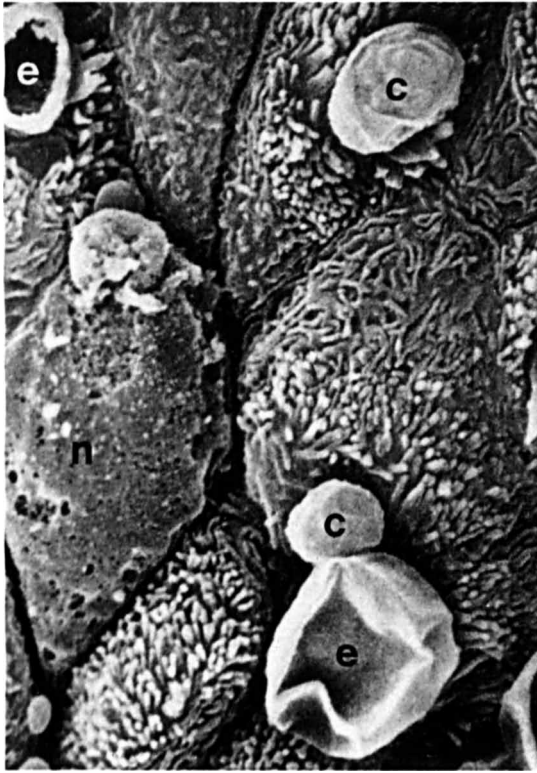
FIG 9: Scanning electron micrograph, ileal Peyer's patch, early cryptosporidial infection. Follicle-associated epithelial cells with cryptosporidia (c). The follicle-associated epithelial cells have irregular folds and microvilli: e, empty parasitic envelope; n, epithelial cell with pores in the luminal membrane, probably a necrotic cell. × 6000

FIG 10: Scanning electron micrograph, colon, convalescent stage of cryptosporidial infection. Note the different length of the microvilli in the surface epithelial cells. × 3000

No comparable studies have been made in ruminants, but such a condition if present, would render the epithelium more susceptible to infections.

Cryptosporidia did not cause extensive fusion of domes with adjacent mucosa in contrast to a combined chlamydia and rotavirus infection (Landsverk 1981b). The fact that follicle size was also unaffected suggests that no major decrease in the IPP lymphopoiesis occurs during cryptosporidiosis. The altered pattern of endocytosis of indian ink may likewise have had little consequence for the lymphopoiesis in the follicles, although subtle qualitative changes may not have been detected with the present technique.

The growth of microvilli in both the FAE and the absorbing epithelium suggests an influence of a soluble factor and not a direct effect of the parasite. Elongation of the microvilli closely adjacent to the parasites has been reported (Pohlenz et al 1978). However, in the present material the change was also found in cases where parasites were rare. Exotoxins

have, so far, not been associated with cryptosporidia, and the cause of the change therefore remains obscure. The consequences of microvillous growth in the FAE seem to include a conformation of luminal membrane that is less compatible with an endocytic function. Endocytosis is apparently one of the main functions of the FAE, its normal lack of rigid cytoskeletal elements and special surface morphology rendering it well equipped for that purpose. The altered pattern of endocytosis of colloidal carbon demonstrated in the present study may thus be due to the growth of microvilli.

A special role in antigen uptake and induction of mucosal immunity has been attributed to the dome over the PP (review, Brandtzaeg 1985). The uptake of carbon particles in the FAE over the IPP demonstrated here confirms an earlier work in lambs (Reynolds and Morris 1983), and may be associated with such an antigen sampling function. Calf rotavirus has likewise been shown to be taken up through endocytosis by the FAE over the IPP (Torres-Medina 1984). In
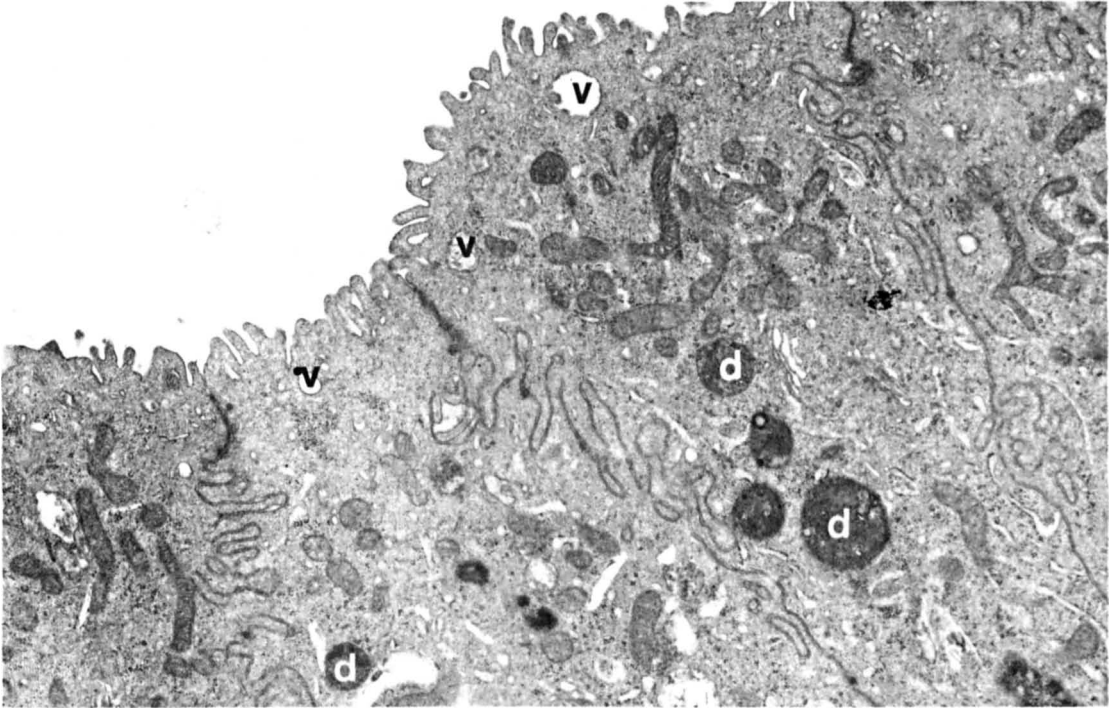
FIG 11: Ileal Peyer's patch, control. The follicle-associated epithelial cells have short microvilli and folds often lacking the prominent core of microfilaments typical for microvilli of absorbing cells. A terminal web is likewise absent. Cytoplasmic vacuoles (v) and dense bodies (d) are seen. × 9000
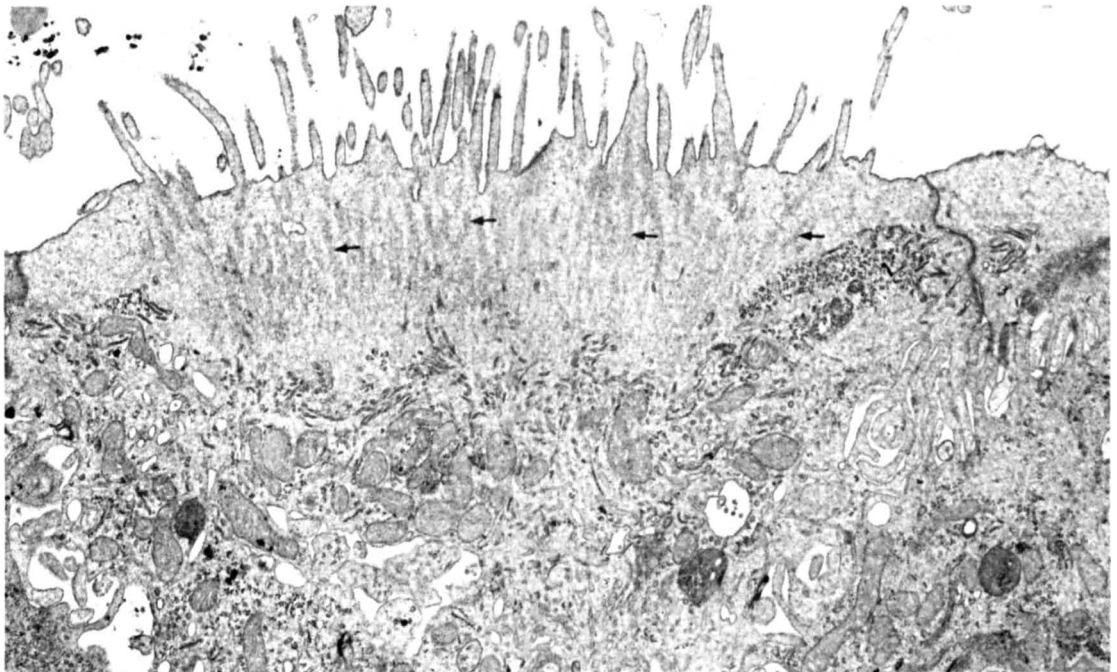

FIG 12: Ileal Peyer's patch, late cryptosporidial infection. The follicle-associated epithelial cells have long microvilli with microfilaments (arrows) inserted deep into the cytoplasm. × 9000

cryptosporidiosis of man a high antibody titre develops (Campbell and Current 1983), probably due to a transport of antigen to the subepithelial tissue allowing an encounter with immunocompetent cells. The cryptosporidia may otherwise be inaccessible to the immune system, hidden as they are in a pocket of the apical epithelial cell membrane (Hampton and Rosario 1966). It is quite possible that cryptosporidial antigen may be transported to the subepithelial tissue by phagocytosis of the FAE over the IPP. The FAE over the IPP has thus been shown to phagocytise $0\cdot6$ μm latex particles (T. Landsverk, unpublished). However, the fact that cryptosporidial antigen was also found in the lamina propria of villi, even proximal to the IPP, suggests that the phagocytising capacity of the FAE was not indispensable to the transport of the antigen.

The mechanism by which cryptosporidial antigen actually was transported into the subepithelial tissue could not be definitely established on the basis of the present observations. Macrophages seemed to play an important role, and in *Giardia muris* infection of mice macrophages were shown to be responsible for trapping of the protozoa in the epithelium (Owen et al 1981). It may nevertheless be suggested that as far as the IPP and cryptosporidia are concerned, there seems to be no exclusive role in antigen uptake for the domes over the IPP.

References

ANGUS, K. W. (1983) *Journal of the Royal Society of Medicine* **76**, 62–70

BRANDTZAEG, P. (1985) *Scandinavian Journal of Gastroenterology* **20**, Suppl. 114, 137–156

CAMPBELL, P. N. & CURRENT, W. L. (1983) *Journal of Clinical Microbiology* **18**, 165–169

CARTER, P. B. & COLLINS, F. M. (1974) *Journal of Experimental Medicine* **139**, 1189–1203

FLEWETT, T. H. (1978) *Journal of the American Veterinary Medical Association* **173**, 538–543

GERBER, H. A., MORRIS, B. & TREVELLA, W. (1986) *Australian Journal of Experimental Biology and Medical Science* **64**, 201–213

HAMPTON, J. C. & ROSARIO, B. (1966) *Journal of Parasitology* **52**, 939–949

HENRIKSEN S. A. A. & POHLENZ, J. F. L. (1981) *Acta Veterinaria Scandinavica* **22**, 594–596

HOHMANN, A. W., SCHMIDT, G. & ROWLEY, D. (1978) *Infection and Immunity* **22**, 763–770

LaBREC, E. H. & FORMAL, S. B. (1961) *Immunology* **87**, 562–572

LANDSVERK, T. (1981a) *Acta Veterinaria Scandinavica* **52** 198–210

LANDSVERK, T. (1981b) *Acta Veterinaria Scandinavica* **22**, 459–471

LANDSVERK, T., LAKESVELA, B., LIVEN, E., LUND, A., ÖDEGAARD, Ö. & ÖRSTAVIK, I. (1983) *Acta Veterinaria Scandinavica* **24**, 431–445

MIYAI, K. (1978) Principles and Techniques of Scanning Electron Microscopy. Ed M. A. Hayat. London, Van Nostrand Reinhold. pp 204–235

OWEN, R. L., ALLEN, C. L. & STEVENS, D. P. (1981) *Infection and Immunity* **33**, 591–601

POHLENZ, J., BEMRICK, W. J., MOON, H. W. & CHEVILLE, N. F. (1978) *Veterinary Pathology* **15**, 417–427

REYNOLDS, J. D. & MORRIS, B. (1983) *European Journal of Immunology* **13**, 627–635

REYNOLDS, J. D. & MORRIS, B. (1984) *European Journal of Immunology* **14**, 1–6

SPRINZ, H., LANDY, M., GAINES, S. & EDSALL, G. (1956) *Federation Proceedings* **15**, 614–615

TORRES-MEDINA, A. (1981) *American Journal of Veterinary Research* **42**, 232–236

TORRES-MEDINA, A. (1984) *American Journal of Veterinary Research* **45**, 652–660

WOODE, G. N., POHLENZ, J. F., GOURLEY, N. E. K. & FAGERLAND, J. A. (1984) *Journal of Clinical Microbiology* **19**, 623–630