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Immunocytological Responses in Porcine Proliferative Enteropathies

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The ileum, colon, and mesenteric lymph nodes of pigs naturally affected by either of the two major forms of proliferative enteropathy, namely, intestinal adenomatosis or hemorrhagic enteropathy, were examined for immunocytological responses to infection by immunocytochemistry, using antibodies directed against elements of the porcine immune system. In both forms, there was mucosal proliferation of immature enterocytes which lacked substantial major histocompatibility complex class II expression and a marked accumulation of immunoglobulin A (IgA) at the apical cytoplasm of affected enterocytes in association with intracellular *Campylobacter*-like organisms. In intestinal adenomatosis, there was only a mild infiltration of $CD8^+$ and $CD25^+$ T cells in the intestinal lamina propria. In hemorrhagic enteropathy, there was a moderate infiltration of $CD8^+$ and $CD25^+$ T cells and IgM^+ B cells in the lamina propria. In rats and humans, villous enterocytes are thought to act as antigen-presenting cells, with major histocompatibility complex class II molecules present on their surface, capable of initiating a T-cell response (particularly of $CD8^+$ T cells) in response to bacterial antigens. Therefore, the selection of immature crypt cells by the intracellular *Campylobacter*-like organisms for entry and multiplication may represent a remarkable microbial adaptation associated with local immunomodulation and enhanced bacterial survival. The accumulation of IgA within affected enterocytes may represent a reduced capability of the cells to process nonspecific IgA or an accumulation of specific IgA.

Proliferative enteropathy or enteritis is a transmissible enteric disease of weaned pigs, hamsters, rabbits, and other species. Consistent features are hyperplasia of immature crypt cells of the intestinal epithelium (particularly those in the ileum and colon) and the occurrence of intracytoplasmic non-membrane-bound *Campylobacter*-like organisms within enterocytes in affected portions of the intestine (8, 27). This organism is probably an as yet uncultured or unidentified species (3, 16, 19). The onset of enterocyte hyperplasia is closely correlated with entry of the organism into enterocytes (18, 28, 29).

In pigs, the disease has two major clinicopathological manifestations, porcine intestinal adenomatosis and proliferative hemorrhagic enteropathy. The first of these is associated with poor weight gain in 2- to 3-month-old pigs, and the latter is associated with melena and/or sudden death in 4- to 5-month-old pigs (26). Animals with both clinical and subclinical adenomatosis frequently recover and demonstrate resolution of the lesions (22). The consistent pathological features of the two forms has suggested that the latter represents a progression of underlying adenomatosis, but the reason for the hemorrhagic component remains unresolved (25, 28). Two other forms, necrotic enteritis and regional ileitis, occur in 4- to 5-month-old pigs, but there is a significant component of secondary infection and mucosal destruction with these forms, making investigation of pathogenesis more complex (26).

Investigation of the immune response to proliferative enteropathy has revealed that specific immunoglobulin A (IgA) and IgM responses to the intracellular organism are detectable in the serum when lesions are present in the intestine, but that the IgG response is weak (7). A preliminary immunocytologic study detected IgA and IgM within the mucosa of affected intestines (9). While the morphology of the early lesions and the various forms of proliferative enteropathy have been described (18), the cellular immune mechanisms involved in the lesions require clarification. We undertook immunohistological studies of affected intestines in an effort to understand the development of the lesions and in particular the difference between the hemorrhagic and adenomatosis forms. Investigations of other mechanisms, such as peripheral blood lymphocyte responses, are hindered by the inability of the organism involved to be readily cultivated in vitro.

MATERIALS AND METHODS

Pathology. Twenty diseased pigs from eight farms and eight age-matched control pigs from three other farms were necropsied. The intestines of each pig were either normal or classified pathologically as intestinal adenomatosis or proliferative hemorrhagic enteropathy on the basis of the occurrence of mucosal hyperplasia, intracellular *Campylobacter*-like organisms within enterocytes, and associated hemorrhage and necrosis (26) (Table 1).

Antibodies. Primary monoclonal antibody (designated IG4; mouse IgG3) to the intracellular *Campylobacter*-like organism had been prepared previously (17). Primary monoclonal or specific polyclonal antibodies to elements of the porcine immune system were obtained as follows. Antibodies to porcine lymphocyte interleukin-2 receptor, CD25 (designated 231 3BT; mouse IgG1), had been prepared previously (1). Primary monoclonal antibodies to porcine lymphocyte subsets CD4, CD8, and macrophages-granulocytes (ATCC HB 147, HB 143, and HB 142, respectively) were obtained from J. R. Lunney, U.S. Department of Agriculture, Beltsville, Md. (12). Antibodies to porcine IgA,

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TABLE 1. Some features of the major findings in pigs affected by intestinal adenomatosis or hemorrhagic enteropathy

Form of disease	Age of pig (mo)	Major clinical signs	Pathology		
			Hyperplasia of ileum mucosa	Hemorrhage into intestinal lumen	Intracellular *Campylobacter*-like organisms
Intestinal adenomatosis	2–3	Anorexia, wasting	+	−	+
Hemorrhagic enteropathy	4–5	Melena, sudden death	+	+	+

IgM, and IgG (polyclonal rabbit antisera; Fc specific) were obtained commercially (Nordic Immunological Laboratories, Tilburg, The Netherlands). Primary monoclonal antibodies to major histocompatibility complex (MHC) class II (total HLA; designated Sw73; rat IgG2a) were obtained from J. Hopkins, University of Edinburgh, United Kingdom (4), and those to porcine MHC class II (SLA-DR; designated MSA3; mouse IgG2b) were obtained from J. R. Lunney (12).

Fluorescein-conjugated secondary antibodies to mouse, rat, or rabbit immunoglobulin were obtained commercially (Sigma Chemical Co., Poole, United Kingdom). Primary and secondary antibodies of rabbit origin were tested for reactivity with *Campylobacter*-like organisms, as natural infection of laboratory rabbits can produce detectable reactions in their sera (8). Only negative antisera were used. All secondary antibody reagents had been affinity purified, and all produced no visible reaction when used alone on any section.

Preparation of tissues. Ileum, mesenteric lymph node, and proximal colon (n = 28, 12, and 20, respectively) were rapidly isolated at necropsy; pieces measuring 1.0 by 0.5 cm were cut, embedded in O.C.T. compound (Miles Laboratories Inc., Elkhart, Ind.) on a piece of cork, and snap frozen in a dry ice-liquid nitrogen slurry. Pieces were stored at −70°C, and then cryostat sections (4 to 6 μm) were cut and air dried at 20°C for 18 h. Separate sections were fixed by a modification of microwave irradiation (10), using 650 W for 50 s, and then stained with Giemsa solution or with hematoxylin and eosin (H&E) and examined. Further fixed sections were immersed in blocking buffer (0.1 M Tris-HCl [pH 7.5], 0.1 M NaCl, 0.1% albumin, 0.5% gelatin) for 1 h at 20°C and then incubated with each primary antibody for 18 h at 4°C. Slides were washed in phosphate-buffered saline, pH 7.4 (PBS), and then incubated with the appropriate secondary antibody for 2 h at 20°C. After being washed in PBS, slides were covered with coverslips mounted in PBS-glycerol and were examined under a fluorescence microscope. All sections were processed with each antibody on several separate occasions and assessed by the same two authors. Preliminary assessment of stained sections of fixed tissues indicated that the lymphocyte marker antibodies did not react on tissues fixed in formalin.

RESULTS

In the normal pigs (2 to 4 months old), cells reactive with the three immunoglobulin and CD4 antibodies were distributed in the follicular areas of intestinal Peyer's patches and lymph nodes, cells reactive with CD4 and CD8 antibodies were distributed in interfollicular and dome areas, and occasional cells reactive with macrophages-granulocytes and CD8 antibodies were located in the lamina propria of the intestine. Cells reactive with HLA/SLA antibodies were apparently macrophages and $CD8^+$ cells in the lamina propria (Fig. 1). There was mild reactivity in the apical cytoplasm of enterocytes, particularly villous cells in the ileum; crypt cells were not stained. No reactions were detected with antibodies to CD25 or *Campylobacter*-like organisms (IG4). In sections stained with H&E, there was a small population of mononuclear and polymorphonuclear leukocytes in the lamina propria of the intestine, similar in distribution to that detected by antibody reactions. In Giemsa-stained sections, occasional mast cells containing positively staining granules were evident.

In all pigs affected with proliferative enteropathy, sections

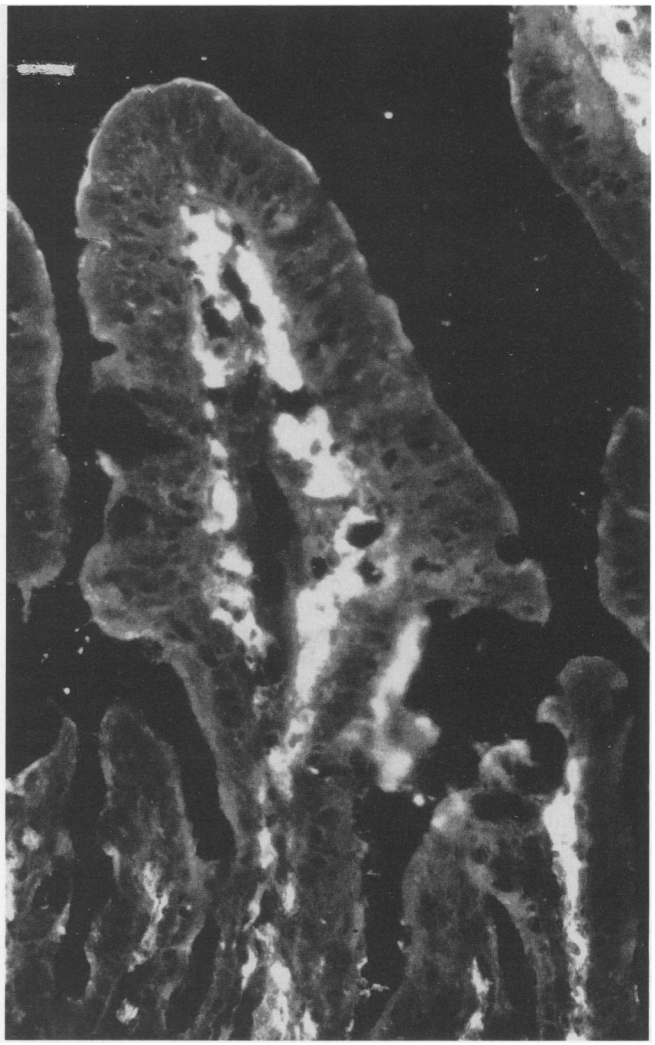

FIG. 1. Photomicrograph of the ileum of a healthy 4-week-old pig, stained by immunofluorescence with MHC class II (total HLA) primary antibody. The apical cytoplasm of villous enterocytes and cells in the lamina propria show mild and marked positive reactions, respectively. Bar = 10 μm.

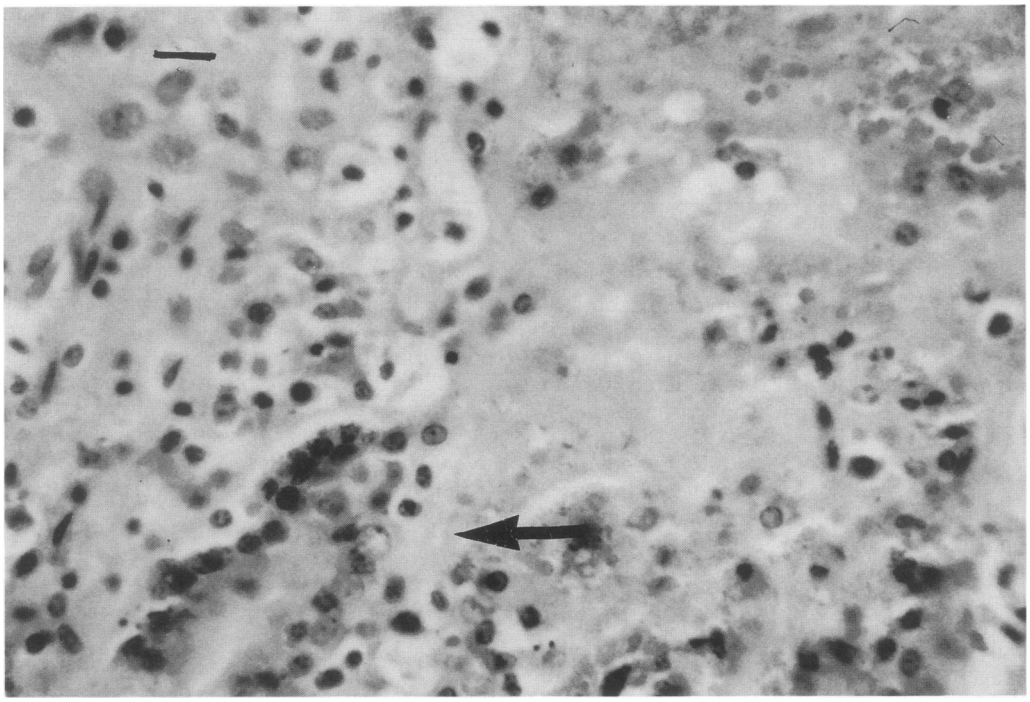

FIG. 2. Photomicrograph of the ileum of a pig affected with proliferative hemorrhagic enteropathy, H&E stain. Shown is the actual site of mucosal hemorrhage (arrow), indicated by close apposition of inflamed mucosa containing mononuclear cells and macrophages to proteinaceous fluid containing erythrocytes in the lumen above. There is also edema and necrosis of the affected mucosa. Bar = 20 μm.

of ileum and colon stained with H&E showed marked hyperplasia of immature crypt enterocytes. In seven 8- to 10-week-old pigs with adenomatosis, there was no evidence of an increase in cellularity in the lamina propria, and the epithelium remained intact. However, in 13 12- to 18-week-old pigs with the hemorrhagic enteropathy form, there was a moderate infiltration of mononuclear lymphoid cells into the lamina propria, with occasional polymorphonuclear leukocytes. There was also marked hemorrhage into the intestinal lumen. In some sections, direct hemorrhage from the mucosa was associated with edema and necrosis of the mucosa (Fig. 2). In Giemsa-stained sections, only occasional mast cells containing positively staining granules were evident. In affected pigs, sections of lymph node showed moderate hyperplasia of follicular, interfollicular, and medullary lymphoid cells.

In the intestines of pigs affected with intestinal adenomatosis, numerous curved bacilli reactive with IG4 antibodies were detected in the apical cytoplasm of proliferating enterocytes and within cells in the lamina propria at the basal mucosa region (illustrated elsewhere [17]). In sections stained with IgA antibodies, numerous vacuolar structures, 3 to 5 μm in diameter, were also detected in the apical cytoplasm of proliferating enterocytes (Fig. 3). These structures appeared to incorporate areas where bacilli were located, and some were associated with bacilli which also stained positively with IgA antibodies. They were not present in normal intestine (Fig. 4). Occasional cells reactive with CD8, CD25, and IgM antibodies were detected in the lamina propria and in the interfollicular and dome areas of intestinal Peyer's patches and lymph nodes. There was a markedly reduced number of enterocytes and lamina propria cells reactive with HLA/SLA antibodies (Fig. 5) compared with normal (Fig. 1) cells, with no fluorescence evident in sections of affected mucosal crypts.

In the intestines of pigs affected with hemorrhagic enteropathy, numerous curved bacilli reactive with IG4 antibodies were similarly detected in the apical cytoplasm of proliferating enterocytes within cells in the basal lamina propria and additionally in the lumens of affected crypts. Macrophages-granulocytes and cell debris reactive with IgA and/or IgM antibodies were also present in these lumens (Fig. 6; see Fig. 4 for comparison). Curved bacilli and occasional vacuolar structures reactive with IgA antibodies were present in proliferating enterocytes in amounts similar to those seen in adenomatosis. Small to moderate numbers of macrophages-granulocytes and cells reactive with CD8, CD25, and IgM (Fig. 6) antibodies were detected in the lamina propria. Moderate populations of cells reactive with CD8, CD25, and HLA/SLA antibodies were distributed in the dome areas of intestinal Peyer's patches and lymph nodes and within the lamina propria of the basal mucosa region (Fig. 7). In comparison, only occasional cells reactive with these antibodies were present in the lamina propria of normal pigs (not illustrated). There was a markedly reduced number of enterocytes reactive with HLA/SLA antibodies in comparison with normal cells, as illustrated in Fig. 5 compared with Fig. 1.

No other alteration to the cellular distribution pattern seen in normal pig sections was detected in either of the disease forms.

DISCUSSION

The consistent features of the proliferative enteropathies are intracytoplasmic *Campylobacter*-like organisms and an

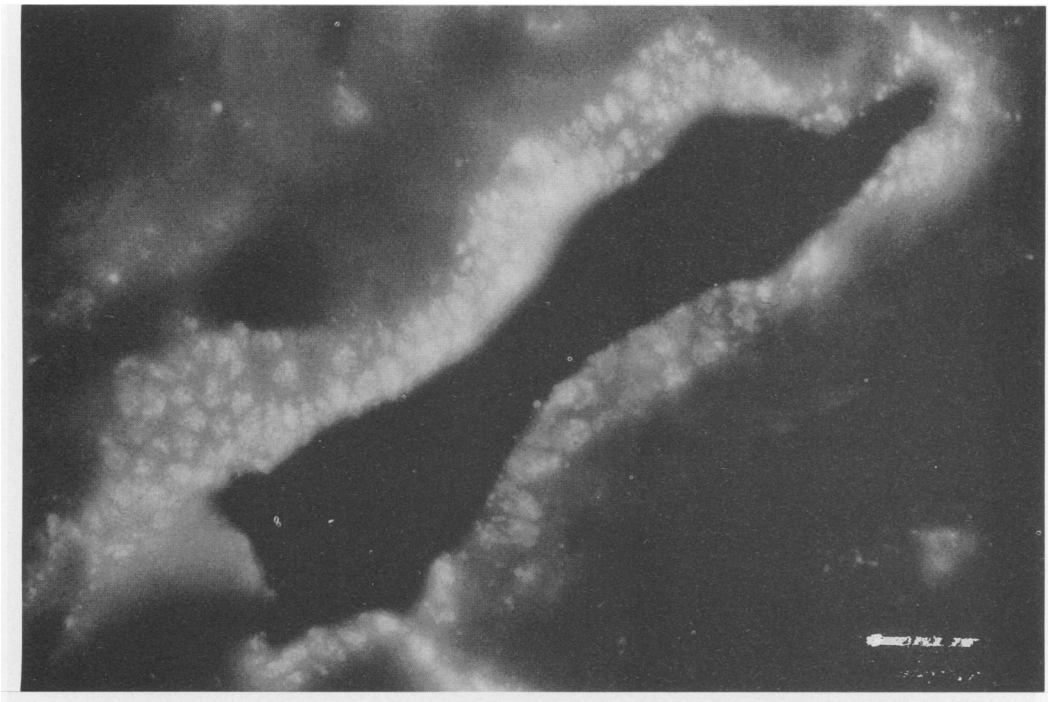

FIG. 3. Photomicrograph of the ileum of a pig affected with intestinal adenomatosis, stained by immunofluorescence with IgA primary antibody. Vacuolar structures are evident in the apical cytoplasm of proliferative enterocytes. Bar = 10 μm.

associated hyperplasia of immature crypt enterocytes. Our study suggests that the immune response in situ initially consists of accumulation of IgA at the position of the cells infected by the organism, with only a mild cellular response of mainly CD8 cytolytic/suppressor T lymphocytes within affected mucosa. In hemorrhagic lesions, a moderate mixed lymphocyte infiltration was evident, with infiltration of CD8 and CD25 T lymphocytes, accompanied by accumulation of

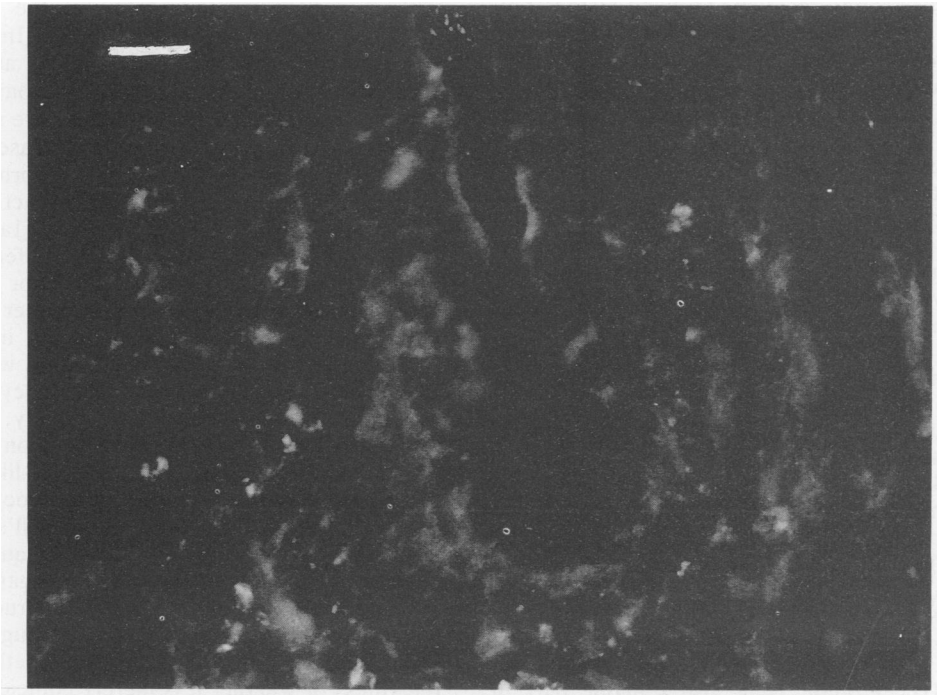

FIG. 4. Photomicrograph of the ileum of a healthy 4-week-old pig, stained by immunofluorescence with IgA primary antibody. Occasional reactive cells are present in the lamina propria, and some staining of the luminal surface of enterocytes can be seen. Crypt enterocytes are not stained. Bar = 10 μm.

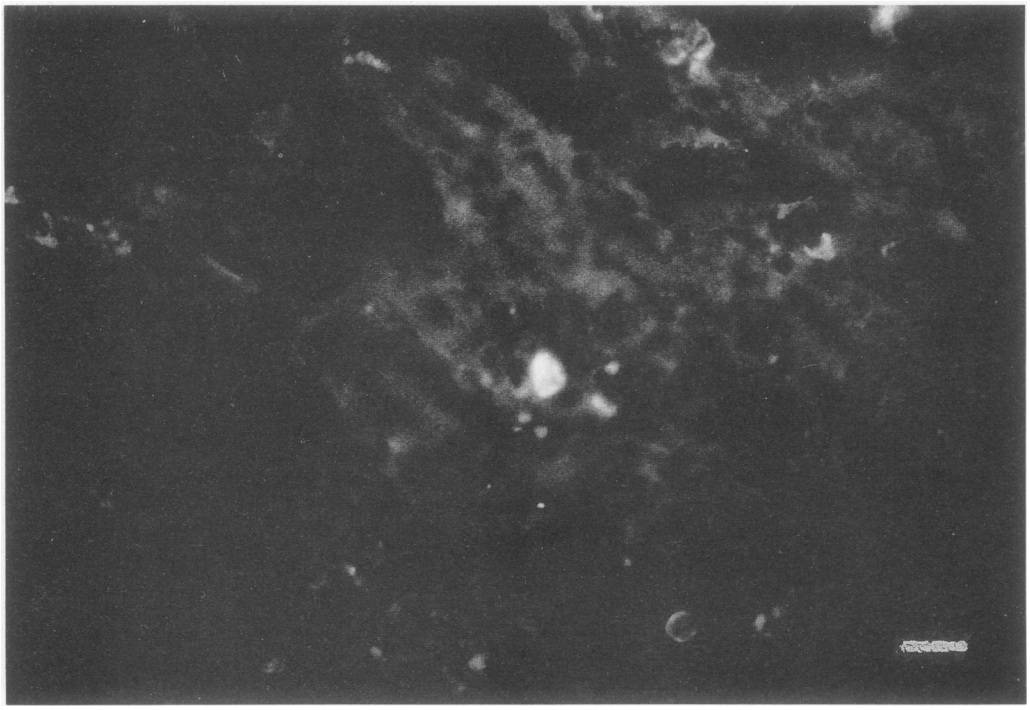

FIG. 5. Photomicrograph of the ileum of a pig affected with hemorrhagic enteropathy, stained by immunofluorescence with MHC class II (total HLA) primary antibody, showing an occasional macrophage-like cell reaction. There is no visible staining of proliferating enterocytes. Bar = 10 μm.

IgA and IgM B lymphocytes and marked cell lysis. The distribution of various leukocyte subsets in the tissues of our normal pigs approximated that found in previous studies (5, 24), as did our IG4 antibody reactions (17) and histopathology of the lesions (18, 25). Electron microscopic and histochemical studies of early lesions of the proliferative enteropathies have established that the presence of intracellular organisms is closely correlated with the proliferation of crypt enterocytes with immature microvillous and enzymatic structures, to the exclusion of mature villous cells (25). Our study suggests that these proliferating cells also lack the substantial MHC class II structures associated with mature villous cells in other species (1a, 15). There are several possible consequences of this alteration to enterocytes.

Villous enterocytes in the human and rat ileum can function as potent antigen-presenting cells, with MHC class II molecules playing an important role in this process (15, 16). Reduction in the numbers of these mature enterocytes could markedly reduce the antigen-presenting capacity of the affected bowel and result in a diminished immune response even if other mucosal antigen-presenting cells, such as macrophages, had some contact with the novel antigens associated with the infection. Previous studies of the immune response to intracellular bacterial infections, such as rickettsial infection of fibroblasts, have suggested that cytolytic T cells (CD8) form a major part of the response to infected cells presenting novel antigens (6, 23). Whereas CD8 cells often respond to antigens presented in conjunction with MHC class I molecules, studies in humans have demonstrated that CD8 cytolytic cells are a major part of enterocyte-associated immune reaction, in conjunction with active MHC class II molecules (13, 14). In the normal pig, CD8 cells usually carry active MHC class II molecules (12) and are present in the lamina propria of intestinal villi. Therefore, the reduction in mature enterocytes and villous structures in the proliferative enteropathies could have a profound effect on the ability of the host to mount a successful cellular immune response. Inflammatory bowel disease in humans has been similarly associated with reduced induction of an active CD8 response (13). Therefore, the proliferative enteropathies may have some pathogenetic mechanisms in common with that disease complex.

Autocrine hormones, such as transforming growth factor β, normally present in villous cells act locally as potent inhibitors of enterocyte proliferation (1a, 20), and the reduced number of villous cells in proliferative enteropathy could reduce local inhibition of crypt cell proliferation, adding to the lesion's progression. Other cytokines such as tumor necrosis factor α and gamma interferon are also potent regulators of immune responses within other intestinal lesions (21), and the mild immunocytological response could affect their activity. Alternatively, some other direct or indirect effect of the bacterial invasion and host response may be involved. The *Campylobacter*-like organism therefore may have selected the crypt enterocyte for its intracellular multiplication as a result of this cell's lack of detectable MHC class II molecules and a consequent weak immune presentation and response, or the organism may actively down-regulate any MHC class II structures present on infected enterocytes, or it may act through a combination of these mechanisms. Whatever the method involved and regardless of whether it is merely coincidental, the location of crypt enterocytes for the organism's habitat may have a positive protective effect on its survival. It is likely that a similar effect occurs during transmissible colonic hyperpla-

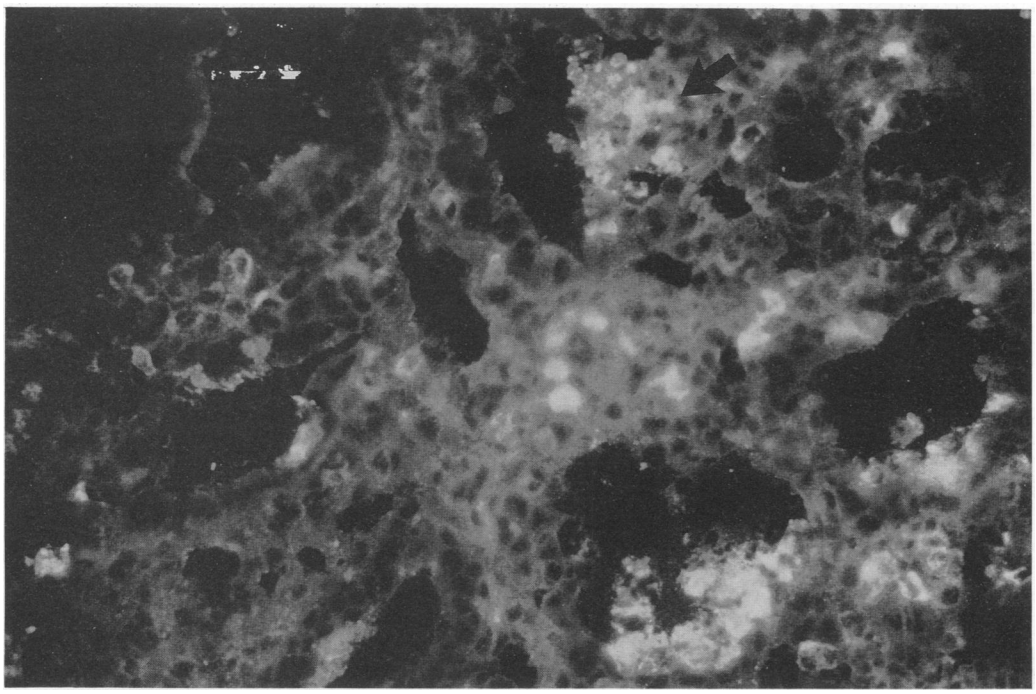

FIG. 6. Photomicrograph of the ileum of a pig affected with hemorrhagic enteropathy, stained by immunofluorescence with IgM primary antibody. There are moderate numbers of positive cells in the lamina propria and positively staining cell debris in the lumen of crypts (arrow). Bar = 20 μm.

sia in mice, due to *Citrobacter freundii*, as there is also a weak immune response in that disease (2).

The moderate CD8 and CD25 response seen in hemorrhagic lesions may have arisen following MHC class I, interleukin, and MHC class II macrophage involvement in the immune response. Even so, the severe, diffuse infection of ileal enterocytes, the loss of cells into the lumen, and the CD8/CD25 and immunoglobulin response present were as-

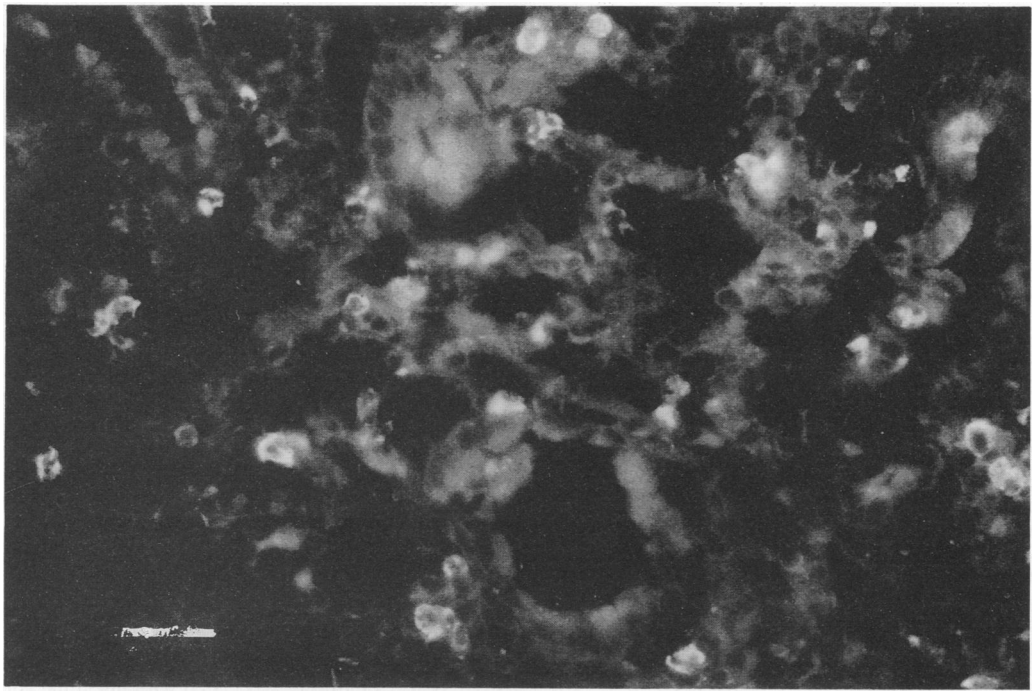

FIG. 7. Photomicrograph of the ileum of a pig affected with hemorrhagic enteropathy, stained by immunofluorescence with $CD25^+$ primary antibody. There are moderate numbers of positive cells in the lamina propria of the basal mucosa region. Bar = 20 μm.

sociated with marked cytolysis and hemorrhage in the proliferative hemorrhagic enteropathy lesions. A feature of many of these lesions is that the marked degree of hemorrhage, often resulting in death, is not associated with marked tissue destruction or mucosal ulceration. It is possible that some alteration to blood vessel formations and viability occurs during altered immune or autocrine regulation. In contrast, it is possible that in mild infections, normal intestinal turnover of cells eventually causes elimination of infected cells, with the recovery of the affected bowel. Development of effective vaccines would therefore have to take into account the immunomodulation which occurs in natural infection.

A previous study of hemorrhagic enteropathy lesions suggested that eosinophils and mast cells could be involved in the pathogenesis of the lesions (11). However, this study and previous morphological studies (25) did not reveal any significant involvement. There was a significant accumulation of IgA and IgM around the site of bacterial infection in the enterocyte, a feature of other gastroenteric infections, including campylobacteriosis in humans (30, 31). The marked accumulation of IgA within enterocytes affected by proliferative enteropathy is, however, a novel phenomenon. It is possible that the presence of the intracellular *Campylobacter*-like organism has a deleterious effect on the enterocyte's ability to process the normal pathway of formation of IgA from secretory component onto the luminal surface, causing an intracellular accumulation of IgA. Alternatively, there could be a buildup of specific IgA, with or without IgA-bacteria complex formation, at the location of infection. The local immunoglobulin response may play some role in presentation of novel antigens; further antigen stimulation studies on lymphocytes isolated from affected animals and on the role of cytokines and IgA in the lesions are in progress.

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