Morphologic studies of the intestinal mucosa of newborn gnotobiotic calves inoculated with a new unclassified virus (Breda virus I)

by

Ahmad Hamdi Mokresh

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INTRODUCTION

The gastrointestinal tract is a natural habitat for many different types of microorganisms including bacteria and viruses (Doughri et al., 1973) which may cause diarrhea. Acute neonatal calf diarrhea or calf scours syndrome is still one of the most important causes of economic losses in both dairy and beef industries. The severity of the economic loss is attributed to high mortality, retardation of growth in surviving animals in affected herds (Bachmann, 1983) and to medical costs for treatment.

The diarrhea syndrome has a worldwide distribution and its complexity has been described by Woode (1978) as follows:

> The proposed causes of the syndrome are many and unrelated and include fungal and chemical toxins, bacteria, viruses, protozoa, environmental factors such as overfeeding, artificial feeding, overpopulation, cold temperature, bad hygiene and colostrum deprivation and the individual animal's susceptibility whether of a phenotypic or a genotypic origin. To this list could be added other agents yet to be discovered, particularly viruses and bacteria with the possibility that the microbial agents concerned have such antigenic variation within a particular genus that the host may be subjected to an almost infinite number of different infectious agents. This is already known for Escherichia coli and probably for the rotaviruses. Further factors to be considered are the efficiency of transfer of the dam's immunity to the neonate and the development of immunological competence of the neonate both in overcoming an active infection and resisting reinfection.

The causative agents of the diarrhea syndrome are incriminated to occur either alone or in combination. The following infectious agents

are often found to be associated with neonatal calf diarrhea: Escherichia coli, Salmonella, Klebsiella, Chlamydia, rotaviruses, coronaviruses, adenoviruses, parvoviruses, caliciviruses and astroviruses. Cryptosporidium sp. has been reported recently as a monoinfectious pathogen in gnotobiotic calves (Pohlenz et al., 1983).

Breda virus was described by Woode et al. (1982) when severe neonatal calf diarrhea was a problem in a beef herd in Iowa for 3 years. The calves developed yellow-white semisolid or watery diarrhea, typical for a virus infection in young calves usually between 3-5 days old and up to 20 days old. While attempting to find the cause, rotavirus and coronavirus were isolated occasionally. However, Woode et al. (1982) found that a special preparation (B276) had large numbers of virus-like particles which had superficial similarity to coronavirus in their morphology but they proved to be different serologically. These particles were named Breda virus after the town of Breda in Iowa.

Breda viruses have two distinct morphologies by electron microscopic study on fecal samples with negative stain (Woode et al., 1982). The spherical particles measured 89 ± 7 nm x 75 ± 9 nm with peplomers 7.6-9.5 nm long. The sausage-shaped virions were 120 ± 15 nm x 32 ± 8 nm with similar peplomers. The latter particles sometimes are curved and resemble a kidney shape. There are 2 Breda virus serotypes which are morphologically similar but are different on the basis of hemagglutination-hemagglutination inhibition test (HAHI) (Woode et al., 1983). In addition, Breda virus is antigenically different from other bovine hemagglutinating viruses.

Although Breda virus I may be confused at a superficial examination with coronavirus, it is morphologically distinct and different by HAHI test and by immunofluorescence. Recently, Breda viruses have been shown to be related antigenically and morphologically to an enveloped RNA virus (Berne virus) in horses (Weiss et al., 1983).

Limited studies on Breda virus-induced lesions in 2 colostrum-deprived calves and in 1 gnotobiotic calf have been described by Woode et al. (1982). The pathological changes were observed in the jejunum, the ileum and the colon. There was villous atrophy, villous fusion, epithelial necrosis and replacement of epithelial cells by immature epithelial cells. Nuclear debris and inflammatory cells were observed in the lamina propria. The surface of the colon had irregular epithelial cells and areas of necrosis. The immunofluorescent positive cells were found on the villi, colonic epithelium and crypts of the lower small intestine and the colon.

As this publication did not include sufficient studies on the pathology and pathogenesis of Breda virus infection in calves, an experiment was planned in a number of gnotobiotic calves by Dr. Pohlenz and Dr. Woode. The animal tissues from these experiments were the basis for the studies presented in this thesis. Initial results were published by Pohlenz et al. (1982), which included data obtained by the author of this thesis and which appear in this thesis. These results confirmed in general the observations made by Woode et al. (1982). However, no studies were made on the ultrastructural cytopathology at that time. Subsequently, the cy-

topathology was described (Pohlenz et al., 1984, in press), which included data obtained by the author and is included in this thesis.

During the first week of life, neonatal animals frequently suffer from a variety of pathogenic enteric infections. These are difficult to control or prevent. For this reason, to determine the pathogenesis and the specific lesions induced by a single agent, it is necessary to produce microorganism-free neonatal animals (gnotobiotic calves).

This study had the following objectives:

1. To classify the morphological changes induced by Breda virus in the gastrointestinal tract of gnotobiotic calves by light and electron microscopy.

2. To locate the virus antigen by immunofluorescence from frozen sections.

3. To evaluate the morphological change for the whole intestine using a methylene blue stain technique.

4. To compare the experimentally-induced lesions with the lesions caused by other enteric viral infections.

LITERATURE REVIEW

There are numerous reports on the pathology of naturally occurring and experimental infections with rotavirus, coronavirus, adenovirus, parvovirus, astrovirus, calicivirus, E. coli and Cryptosporidium sp. For the purpose of this review and for comparison with Breda virus infection, only those agents whose pathogenesis has been studied in gnotobiotic calves will be included.

Pathophysiology of Diarrhea

The anatomy of the small intestine provides a huge surface for transepithelial movement of water and electrolytes. These fluids circulate between blood and intestinal lumen. Besides fluids originating from gut secretion, there are salivary, gastric, biliary and pancreatic secretions contained in the intestinal lumen (Whipp, 1978). Secretory fluxes and absorptive fluxes occur at the same time across the mucosa, but the absorptive fluxes exceed the secretory ones which results in net absorption (Moon, 1978). These fluxes in both directions occur by passive diffusion through pores in the epithelial cell junctions. Only small amounts of the fluxes in either direction require active transport. According to Whipp (1978), the tight junctions of intestinal epithelial cells are cation selective and are more permeable to cations than ions. Argenzio (1978) reported similar pathways for the absorptive function of the colon. In addition to fluid transport, villous epithelial cells of the small intestine have digestive and absorptive functions. Carbohydrates, proteins and

fat are digested in the small intestine. When ingesta reaches the ileal-cecal valve it should not contain undigested residues (Bywater, 1980). For instance, lactose which is contained in the diet of young animals is not absorbed unless it is hydrolyzed to glucose and galactose by the enzyme lactase in the brush borders of epithelial cells.

In general, the cause of death in animals suffering from viral or bacterial diarrhea is the result of excessive loss of water and electrolytes leading to dehydration, acidosis and shock (Whipp, 1978). Different enteropathogenic microorganisms have been shown experimentally to produce these events. Various mechanisms of intestinal functions are involved when fluid loss occurs (Moon, 1978). The most important ones are hypersecretion, changes in mucosal permeability, changes in intestinal motility and disturbance of digestive and absorptive functions.

Diarrhea may occur due to increased total secretion beyond absorptive capacity or from absorptive impairment (Moon, 1978). Intestinal secretion is controlled by cyclic adenosine monophosphate (CAMP) and intestinal secretion may also be related to increased levels of intracellular cyclic guanosine monophosphate (CGMP) (Whipp, 1978). The permeability of the small intestine to passive movements of sodium and water is highest in the duodenum and least in the ileum (Argenzio and Whipp, 1980). Intestinal inflammation results in increased hydraulic pressure with increased pore size of mucosal membrane (Moon, 1978) and if the exudative material exceeds the absorptive capability of the intestine, diarrhea will result. Digestion and absorption of ingesta which passes through the intestinal tract re-

quire a time of contact between ingesta and mucosa. Argenzio (1978) stated that a delay in transit time is necessary for the colon to perform its functions of microbial digestion and absorption of water and electrolytes. Increased fluidity and volume of intestinal contents may result in decreased transit time of the ingesta and decreased contact time with the mucosa.

Both maldigestion and malabsorption occur in diarrhea. Maldigestion may result from digestion failure due to pancreatic atrophy or intestinal abnormality (Moon, 1978). Destruction or loss of villous epithelial cells with their brush borders and their associated digestive enzymes will result in malabsorption and maldigestion (Bywater, 1982; Berschneider and Argenzio, 1982). This means that nutrients are not or are insufficiently digested and fluids are insufficiently absorbed in the small intestine. This incompletely digested intestinal material will enter the large intestine where it disturbs the large intestinal functions. These materials in the colon produce osmotically active metabolites which induce hyperosmolality in the lumen. The normal fermentation products are hydrogen ion, volatile fatty acids, and carbon dioxide (Argenzio, 1978). The presence of fermented metabolites increases lumenal acidification and hyperosmolality which tends to hold water in the lumen and draws water from the circulation into the lumen, thus contributing to diarrhea (Moon, 1978; Bywater, 1980). Continuous loss of water and electrolytes in diarrhea results in dehydration (Bywater, 1982). The degree of dehydration is correlated with the clinical signs (Bywater, 1980). The manifestations are

mild depression and decreased urine output when 5% of body weight is lost; sunken eyes, tight skin, dry mouth and further urine reduction when 6-8% is lost; cold extremities and recumbancy when 9-11% is lost and shock and death when 12-14% is lost. Hypoglycemia occurs in acute severe diarrhea and it is interpreted to cause the weakness, lethargy, convulsions and coma (Lewis and Phillips, 1978). Hypoglycemia is due to impaired glucose absorption through damaged mucosa (Berschneider and Argenzio, 1982). Bywater (1970) found that oral fluid therapy consisting of glucose, amino acids and sodium is necessary for survival of animals suffering from diarrhea. Fluid lost in diarrheic calves contains sodium, potassium and bicarbonate (Bywater, 1982). These losses result in a decline of the levels of these ions in the plasma. A total loss of body potassium occurs although the animals present as hyperkalemic (Berschneider and Argenzio, 1982). High concentrations of hydrogen ions in the extracellular fluid causes hydrogen to diffuse into the cells forcing the potassium ions out. The potassium leaving the cells enters the plasma and causes hyperkalemia despite the overall deficit (Bywater, 1982). Acidosis results from loss of bicarbonate in feces and it occurs in association with insufficient renal excretion of hydrogen ions and absorption of bicarbonate (Moon, 1983).

Rotavirus Infection

Rotavirus was first described as a viral infection causing diarrhea in neonatal calves by Mebus et al. (1969) and called reovirus-like. Sub-

sequently, rotavirus was recognized as a new genus in the family reoviridae (Matthews, 1982). Woode et al. (1976) found that rotaviral particles varied between 50-69 nm in diameter depending on the presence or absence of the outer capsid layer. The virus genome consists of 11 segments of double stranded RNA with core diameter of 30-36 nm (Tzipori, 1981; Mebus, 1975). The outer layer of the virus contains glycoprotein which is responsible for serological specificity (Derbyshire and Woode, 1978). Rotaviruses have been isolated from a wide range of mammalian species including children, calves, pigs, lambs, mice, foals, monkeys, deer, pronghorn antelope and rabbits; serological evidence of rotavirus infection was obtained from guinea pigs and goats (Woode et al., 1978). All viral isolates are morphologically indistinguishable and their antigenic cross reactivity is associated with the inner capsid layer (Derbyshire and Woode, 1978).

Rotaviruses have affinity for absorptive epithelial cells of the small intestine after oral entry. Middleton (1978) stated that enterocytes of the small intestine are the site of viral replication in pigs and humans infected with human rotavirus. This infection in humans and pigs was restricted to the duodenum and upper jejunum; however, a viral antigen has been demonstrated in fatal cases throughout the small intestine. Mebus (1976) suggested from his experiment that after oral inoculation the columnar epithelial cells on the upper two-thirds of the villi in the upper part of the small intestine became infected and the infection rapidly progressed caudally. Immunofluorescent positive cells were not

observed a short time after the onset of diarrhea. This concept was not supported by Pearson et al. (1978b) who found that specific rotavirus immunofluorescence was observed in epithelial cells in the intestine of infected calves which excreted the virus for 48 hrs. Mebus (1975) postulated that the virus redirected the epithelial cell function from absorption to virus production. He speculated that there was accelerated migration of infected cells up to the villous tips. These cells were sloughed and replaced by cuboidal cells. Woode et al. (1978) speculated that the loss of the absorptive cells of the small intestine or the loss of function of these cells in early infection resulted in a malabsorption syndrome: this was supported by the D-xylose absorption test results.

The clinical signs vary from mild to severe illness with death under field conditions (Woode and Bridger, 1975). The morbidity in severe outbreaks is nearly 100% and the mortality ranges from 0-50% (Mebus et al., 1971). Most of the reports indicate that rotavirus infection occurs in the first few weeks of life and as early as one day of life. Apparently, there is no age resistance to rotavirus infection (McNulty, 1978). Tzipori (1981) isolated the virus from infected calves under field conditions up to 8 weeks of life. Woode et al. (1978) suggested that the incubation period ranged from 15 to 24 hrs and even to 96 hrs. Mebus (1976) observed that clinical signs consisted of depression, anorexia, a few strings of thick saliva hanging from lips and diarrhea. The diarrhea in milk diet calves is profuse and white-yellow (Woode et al., 1978).

Stair et al. (1973) found that the pathological changes in experi-

mentally-infected calves were found throughout the small intestine and that the upper small intestine was infected first. The villous absorptive cells have been shown to be the natural site for viral infection and replication; however, rotavirus was isolated from lungs and mesenteric lymph nodes of experimentally-infected gnotobiotic calves (Mebus et al., 1971). Mebus (1976) also isolated the virus from jejunal and ileal mesenteric lymph nodes. Generally, the lesions were observed in the upper half to one-third of the villi which were covered with low columnar to cuboidal or squamous epithelial cells (Mebus et al., 1971; Tzipori, 1981). Denuded villous tips have been observed in gnotobiotic calves (Mebus, 1976). Virions were not detected by electron microscopy in the immature epithelial cells that replaced the infected mature epithelium (Stair et al., 1973). The immature cells lacked microvilli and digestive enzymes. The microvilli of the infected cells were shortened and irregular in length, space and shape. Shortening and stunting of villi were reported to occur in rotavirus-infected colostrum-deprived calves (Woode et al., 1974). These authors also observed that the lamina propria of the intestine became thickened and increased its cellularity with the presence of pyknotic mononuclear cells. Stunted and fused villi were predominant lesions in severely affected colostrum-deprived calves (Logan et al., 1979) and pigs (Pearson and McNulty, 1977). Clusters of viral particles were observed in dilated cisterna of endoplasmic reticulum which are proposed to be the site of viral replication (Mebus, 1976). The intestinal structure will return almost to normal in about

8-10 days after the onset of the disease (Flewett and Woode, 1978). Goblet cells in the small intestine were not infected (McNulty, 1978). In calves, there was no evidence of rotavirus infection in crypts or large intestinal epithelial cells. However, a few immunofluorescent cells were found in the large intestine of rotavirus-infected lambs (Snodgrass et al., 1977).

Coronavirus Infection

Bovine coronavirus was first described in Nebraska by Stair et al. (1972) and it was further characterized by Sharpee et al. (1976). The virus has a diameter of 70-80 nm in infected monolayer cells or from infected intestinal epithelial cells (Doughri et al., 1976; Mebus et al., 1973). The intact virion has an envelope with 17-24 nm long projections (Bridger et al., 1978). Coronavirus has also been reported to cause enteric disease in pigs (Tajima, 1970), sheep, deer (Tzipori et al., 1978; Durham et al., 1979), dogs (Keenan et al., 1976), turkeys (Cheville, 1975) and horses (Durham et al., 1979). The name coronavirus was proposed to describe the wide-spaced, petal-shaped projections which resemble the solar corona (Almeida et al., 1968). The coronavirus group has major properties such as characteristic surface structure, size, RNA content, presence of an essential lipid envelope and low particle density. The pathology of transmissible gastroenteritis (TGE) has been studied extensively (Hooper and Haelterman, 1969) and has been used as a model for all studies in the pathogenesis of animal enteric coronavirus infection.

The pathogenesis of bovine coronavirus infection was reported by

Doughri et al. (1976) and Storz et al. (1978). Coronavirus has been shown to infect and alter the mature absorptive epithelial cells. Viral uptake has been shown to occur as the virions interacted with the glycocalyx and adsorbed to microvilli by fusion of the viral envelopes with plasmalemma of the microvilli of absorptive epithelial cells. Another route of viral entry is through intercellular spaces. Virions in this case interacted with lateral cell membranes of adjacent epithelial cells. The infected cells underwent cellular changes during viral morphogenesis and viral production. Viral release occurred as infected cells had fragmentation and lysis of the apical plasmalemma with subsequent flow of the cytoplasmic content into the gut lumen. Vacuoles containing virions fused with apical plasmalemma resulting in release of their contents into the gut lumen or the entire cells sloughed (Mebus, 1978).

The incubation period in gnotobiotic calves inoculated with corona virus varied from 19 to 24 hrs (Mebus et al., 1973; Mebus, 1978). De Leeuw et al. (1982) reported that virus shedding in diarrheic calves varied from 4-7 days. Clinical signs of coronavirus infection in calves is dependent on the animal's age and viral strain as shown by De Leeuw et al. (1982). Calves most often become infected naturally at 7-10 days of life (Mebus, 1978). Bridger et al. (1978) observed that 35-day old calves excreted the virus in their feces without diarrhea. Thus, there may be an age resistance or decreased susceptibility with age. Mebus et al. (1973) observed that experimentally-infected gnotobiotic calves with a diarrhea were depressed, anorexic and passed liquid yellow feces which were oc-

casionally associated with crud-milk material and mucus. Mebus et al. (1975b) observed that gnotobiotic calves had severe depression with strings of saliva hanging from their mouths at 15 hrs post-inoculation. Diarrhea may last about 4 days in infected calves (Bridger et al., 1978).

At necropsy, Doughri and Storz (1977) observed that colostrum-deprived calves inoculated with coronavirus had petechiae in the abomasal mucosa. The lesions induced by coronavirus infection have been reported to be most prominent and are consistently found in the middle and lower small intestine (Bridger et al., 1978) and to lesser degrees in the large intestine (Doughri and Storz, 1977). Storz et al. (1978) reported that the virus infected mature absorptive epithelial cells and goblet cells. The virus has occasionally been shown to infect epithelial cells at villous bases as well as fibroblasts and endothelial cells of small blood capillaries of the lamina propria. Mebus (1978) found that at the early onset of diarrhea, small and large intestinal mucosa appeared histologically normal; however, the cells contained large quantities of viral antigens. No lesions have been found in the duodenum of infected calves (Takahashi et al., 1980). Mebus et al. (1975b) found that after 34 hrs of diarrhea the duodenum had short villi, many of which were covered by cuboidal epithelial cells and that some tips had squamous epithelial cells. Bridger et al. (1978) found no changes in the villous height although some thick villi with cuboidal epithelial cells were observed. Moderate increases in lamina propria cellularity, scattered pyknotic nuclei and dilated lacteals with macrophages, some of which had fragmented nuclei, have

been reported (Mebus et al., 1973; Mebus et al., 1975b). Doughri and Storz (1977) and Bridger et al. (1978) reported similar lesions in the jejunum as well as in the ileum. The villi were short and broader than normal. The epithelial cells were cuboidal and they were squamous on some tips (Mebus et al., 1975b). Some villi underwent different stages of degeneration and necrosis (Doughri and Storz, 1977). Moderate to extensive villous fusion has been reported by Mebus et al. (1975b) and Doughri and Storz (1977). The latter observed that the crypts were hyperplastic and had increased numbers of mitotic figures. Some of these crypts were dilated and had flattened epithelial cells. The lumens contained inflammatory exudate and cellular debris. In addition, Mebus et al. (1975b) reported that in the ileum the lamina propria beneath detaching epithelial cells had numerous cells in which nuclei were pyknotic. Mebus et al. (1973) found that scattered cells in the lamina propria of the ileum fluoresced only with conjugate for coronavirus. The large intestine of one infected calf, killed 34 hrs after onset of diarrhea, lacked colonic ridges (Mebus et al., 1975b). The epithelial cells in many areas varied from low columnar to cuboidal or squamous in appearance. Some epithelial cells underwent necrosis and detachment and were sloughed into the lumen (Doughri and Storz, 1977). Early after the onset of diarrhea (Mebus et al., 1973) and later at 73 hrs (Mebus et al., 1975b), the columnar epithelial cells were positive for immunofluorescence. These authors found that some dilated crypts were lined by low cuboidal cells and contained sloughed degenerated cells. Doughri and Storz (1977) re-

ported similar changes in the crypts. Mebus et al. (1975b) found the colonic lamina propria had numerous pyknotic cells, some of which were in dilated lymphatics. Goblet cells were less in number in the colonic surface and crypts (Mebus et al., 1973; Bridger et al., 1978). These authors found that surface and crypt epithelium and mesenteric lymph nodes fluoresced when stained with anticoronaviral conjugate. In gnotobiotic calves killed 3 hrs after onset of diarrhea, mesenteric lymph nodes were severely depleted of lymphocytes (Mebus et al., 1973). The subcapsular sinuses and medullary sinuses contained neutrophils and macrophages, many of which were karyorrhectic. Many cells immunofluoresced for coronavirus antigen in the upper and middle mesenteric lymph nodes.

Astrovirus Infection

In 1974, Woode et al. observed in Britain that feces from one calf out of five which were infected with coronavirus contained small viral particles with a diameter of 27-32 nm. These particles were detected by electron microscopy and did not appear to produce lesions. In 1978, Woode and Bridger found small round viruses of the same size in 3 calves from 3 herds with a history of diarrhea problems. They described that this agent was similar to human astrovirus. These 2 viruses have been shown by immunofluorescence to have different antigens. A serological survey confirmed that an astrovirus agent was common in bovine populations (Woode and Bridger, 1978). Snodgrass et al. (1979) could demonstrate by immunofluorescence that astroviruses from lamb, calf and human origin were not serologically related.

The name astrovirus was suggested by Madeley and Cosgrove (1975a). In their work, they found that 19% of 121 fecal samples from children with gastroenteritis contained viral particles. These particles were uniform and had a diameter of 28 nm. The surface of the viruses had a roughly star-shaped configuration and for this reason they were named astroviruses (Madeley and Cosgrove, 1975b).

Astroviruses were detected in babies under 2 years of age (Madeley et al., 1977; Madeley and Cosgrove, 1975a). Woode and Bridger (1978) isolated the virus from calves less than 7 days old and they suggested that the incubation period was 1-3 days. It is possible that the astrovirus is a primary pathogen and it is commonly associated with other viral infections such as rotavirus and coronavirus (Woode and Bridger, 1978). Bridger et al. (1984) found that 21-49 day old gnotobiotic calves inoculated orally with astrovirus SRV-1 had no clinical signs even though astrovirus was excreted in the feces from day 2 to 7 days after the inoculation. Woode et al. (in press) stated that astrovirus-infected calves remained clinically normal, although the feces became yellow and slightly soft. This confirmed earlier observations (Woode and Bridger, 1978) that the bovine astrovirus alone does not produce diarrhea or clinical signs of the disease. Snodgrass et al. (1979) found in their experiment on astrovirus-infected gnotobiotic lambs that this species developed diarrhea at 44-48 hrs post-inoculation and virions were detected in feces at 38-48 hrs post-infection. Feces in lambs changed from dark brown to very loose and yellow in the early stage of the disease.

Originally, there was work done on astrovirus and pathogenesis in calves as bovine astrovirus was considered to be nonpathogenic since no clinical signs were observed (Woode and Bridger, 1978). In these SRV-2 experimental infections, some decrease in villous height to crypt depth ratio was observed. In lambs, Snodgrass et al. (1979) found that astrovirus infected the apical two-thirds of the midgut villous epithelial cells and subepithelial macrophages in the early stage of infection, 23 hrs post-infection. Infected epithelial cells became cuboidal and many had pyknotic nuclei. In the later stage of the disease, villous atrophy was noticed in the midgut and the ileum. Gray et al. (1980) found macrophages containing viral particles in lysosome-like organelles in the lamina propria of the small intestine of infected lambs. Recently, astrovirus has been shown experimentally in gnotobiotic calves to cause degeneration of ileal dome epithelial cells and no other lesions at sites in the small or large intestine (Woode et al., in press). This change was observed with astrovirus infection alone or in combination with either Breda virus II or rotavirus.

Calici-like Virus Infection

(Newbury Agent)

Calici-like viruses have been reported recently in several species of animals. Madeley and Cosgrove (1976) first reported typical calicivirus, 30 nm in diameter, in fecal samples taken from babies during a stool survey of Glasgow children. Woode and Bridger (1978) suggested the name Newbury agent and described a 33-nm particle which resembled a ca-

licivirus in respect to size and of the lacelike appearance of their edges. Newbury agent did not infect calf kidney cell culture but did cause diarrhea and clinical illness in gnotobiotic calves. The infection was judged by histological examination, by the malabsorption of D-xylose in experimental animals and by the ability of the virus to produce diarrhea in 12 calves. In 1980, Bridger found a calicivirus, 37 nm, in association with piglet diarrhea of 3-week old and very young pigs. Experimentally, this virus produced clinical disease in piglets. In 1984, a new isolate, Newbury agent SRV-1, was described by Bridger et al. This isolate was antigenically distinct from a morphologically similar bovine calicivirus. They found that infection with this isolate appeared to cause more severe changes than those produced by Newbury agent SRV-2, but this virus appeared to have similar pathogenecity. The viral particles consisted of a reticulate appearance with capsomers projecting from their outer edges (Woode and Bridger, 1978). Occasionally, an outer rim 7-8 nm wide was observed by negative stain. Woode and Bridger (1978) also described that the particles occurred singly or in groups varying in numbers from few to several hundred. Within these groups, smaller particles, about 18 nm in diameter, were sometimes observed.

Woode and Bridger (1978) found that calicivirus was capable of producing diarrhea in gnotobiotic calves up to at least the 5th week of life. Experimentally, the fecal-oral route of transmission was successful with diarrhea commencing 24-72 hrs post-inoculation. The incubation period varied from 1 to 5 days (Woode and Bridger, 1978; Bridger et al., 1984).

Mild diarrhea with fecal color changing to yellow and some softening is characteristic of the infection in calves less than 8 days of age. However, profuse yellow diarrhea is characteristic of infected calves more than 16 days old (G. N. Woode, Department of Veterinary Microbiology, Iowa State University, personal communication, 1984). In severely affected calves, blood was seen in the feces on days 15 and 16 after experimental infection (Bridger et al., 1984). Anorexia developed in 63% of infected calves and lasted for 4 days. Viral particles were identified in feces the day before or on the day when clinical signs appeared.

Calves inoculated with Newbury agent had microscopic lesions confined to the small intestine (Woode and Bridger, 1978). These lesions were restricted to the jejunum and the anterior ileum (Hall et al., 1982). Woode and Bridger (1978) found that the villi became short and thick with increased cellularity of the lamina propria. Although most villi had columnar epithelial cells with normal brush borders, some villi had lost their epithelial cells on the upper third and some were covered by cuboidal cells. Hall et al. (1982) observed that early lesions consisted of swollen enterocytes with abnormal shaped microvilli. Sloughed cells, shortened villi with crypt hyperplasia and cuboidal or flat epithelial cells were seen. Later, in the course of the disease, stunted, fused and denuded villous tips were observed along with degenerated macrophages in the lacteals and nuclear debris in the lamina propria. Both authors were not able to detect the viral particles by transmission electron microscopy. In 1978, Woode and Bridger did not observe immunofluorescence in the small

intestine of infected calves with antiserum to Newbury agent; however, Hall et al. (1982) detected immunofluorescence in cells on villi of calves killed 0.5-3 days post-inoculation. This infection was associated with marked loss of lactase activity in the jejunum. No microscopic lesions were observed in the stomach, large intestine, liver, kidney or lung (Woode and Bridger, 1978).

Escherichia coli Infection

Escherichia coli (E. coli) are gram negative bacterial microorganisms. The pathogenesis of these bacteria has been shown to depend upon expression of 2 virulence factors (Isaacson et al., 1978). First is the production of pilli which facilitate mucosal attachment. The second is the production of 2 enterotoxins, heat stable (ST) and heat labile (LT) toxin. These stimulate the small intestine to secrete water and electrolytes through increased production of adenylcyclase (Merritt, 1980). The results are hypersecretion of electrolytes, primarily Na^+ and Cl^- and water.

Runnels et al. (1980) and Moon (1983) described the intestinal tract of conventional and gnotobiotic calves infected with E. coli as having little or no structural damage. Moon (1983) stated that it is not known if absorptive cells are damaged by adherent E. coli or whether they are damaged due to circulatory and metabolic changes resulting from diarrhea. In contrast, Bellamy and Acres (1979) described lesions experimentally induced in colostrum-fed calves orally inoculated with enterotoxigenic E. coli. The marked lesions were in the jejunum and iluem.

The lesions were characterized by short, stunted villi which were covered with cuboidal epithelial cells and sloughing cells at villous tips. Neutrophils were predominant above the dome areas of Peyer's patches. Pearson et al. (1978a) reported lesions in the iluem of E. coli-infected colostrum-deprived calves. The lesions consisted of villous atrophy and villous fusion. Multifocal fibrino-ulcerative ileitis was observed in one calf. Bellamy and Acres (1979) and Pearson et al. (1978a) found gram negative rod-shaped bacilli adhered to the absorptive cells. They also considered the crypts normal. Bellamy and Acres (1979) found no histological changes in the abomasum or the colon of the infected calves. They believed that the local accumulation of neutrophils above the dome areas which have been seen in natural or experimental E. coli infection are the result of E. coli infection and that these changes are not observed with any of the other agents involved in neonatal diarrhea. In these studies, gnotobiotic calves were not used and it is possible that the animals were infected with a virus for which the authors did not have the technique for viral demonstration.

Cryptosporidium Infection

Cryptosporidium sp. is a parasitic coccidium and represents a genus out of the family Cryptosporiidae. This small parasite with a diameter of 1-5 microns, depending on the stage of life cycle and growth, develops in the intestinal host in the microvillous brush border of epithelial cells (Pohlenz et al., 1978b). Recent investigations have shown that this parasite has a worldwide distribution and occurs in many mammalian and avian

animal species. Since 1971, cryptosporidium has been shown to occur in association with diarrhea in the bovine species. Pohlenz et al. (1983) demonstrated experimentally in gnotobiotic calves that Cryptosporidium sp. is a monoinfectious parasitic organism.

Infection resulting from this parasite interferes with digestion, absorption and secretion predominantly in the small intestinal mucosa. Snodgrass et al. (1980) reported several stages of Cryptosporidium sp. embedded in the microvilli of the epithelial cells. The absence of microvilli at the attachment site was observed by Meuten et al. (1974) and Pohlenz et al. (1978a). The destruction and absence of microvilli leading to the lack of digestive and absorptive function may enhance diarrhea (Pohlenz et al., 1978b; Pohlenz et al., 1983).

Cryptosporidial infection occurred in 5-12 day old calves (Meuten et al., 1974; Morin et al., 1976; Pohlenz et al., 1978a; Moon et al., 1978; Anderson, 1981). Panciera et al. (1971) documented an infection with cryptosporidium in an 8-month old animal. Experimentally, the infection was induced in newborn calves and one 3-week old gnotobiotic calf (Pohlenz et al., 1983). Tzipori et al. (1983) showed with experiments in specific pathogen-free calves that the incubation period was 2 to 3 days and clinical illness varied from 5 to 10 days. In naturally occurring infection, Meuten et al. (1974) reported a 14-day old calf with a history of anorexia, weight loss and diarrhea of 10 days duration. This calf was also emaciated and dehydrated. The clinical signs in 2-3 day old specific pathogen-free calves and in gnotobiotic calves inoculated with cryptosporidium were similar to those observed in field cases (Poh-

lenz et al., 1983; Tzipori et al., 1983). The feces were yellow and watery to white and pasty and often contained blood, bile, mucus and undigested milk.

Pathological changes induced by Cryptosporidium sp. have been described in naturally occurring infections either alone or in association with other pathogens. The lesions were also described in gnotobiotic calves and specific pathogen-free calves. In natural infection, Cryptosporidium sp. was observed in the lower small intestine, in the jejunum (Anderson, 1981; Willson and Acres, 1982) or in the jejunum and ileum (Meuten et al., 1974; Pohlenz et al., 1978a). The infection of the cecum and the colon was also observed (Anderson, 1981; Pohlenz et al., 1978a). Experimentally, Pohlenz et al. (1983) induced the infection in gnotobiotic calves. There was villous atrophy, fusion of villi and crypt hyperplasia in the small intestine. These changes were more pronounced at day 5 post-infection than on day 3. Tzipori et al. (1983) found the changes were more severe in the ileum of infected specific pathogen-free calves. The ileal villi were covered by cuboidal epithelial cells and changes were seldom observed in the proximal small intestine. The organisms were found in all sites of the small and large intestinal epithelial cells of different calves and only in crypts of the large intestine of some calves.

MATERIALS AND METHODS

Gnotobiotic calves were used in these experiments to describe the pathogenesis of Breda virus I infection. Dr. G. N. Woode, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, was responsible for the preparation of the animals, the provision and assay of the virus inoculum, virus isolation and immunofluorescence of the intestinal tissue sections. Dr. J. Pohlenz, Department of Veterinary Pathology, Iowa State University, was responsible for the autopsies performed with the assistance of the author. The author studied histopathology and ultrastructural cytopathology with Dr. Pohlenz.

Calves

The experiment was performed on six gnotobiotic calves (GC). Two of these calves served as control calves. The experimental GC were obtained by the open caesarean technique as described by Matthews et al. (1981) and kept in a plastic isolator. During the experiment the calves were fed a diet consisting of sterilized and reconstituted evaporated cow's milk.

The inoculated calves were GC 14, GC 16, GC 18 and GC 20 (Table 1). They were intranasally instilled within 1-2 hours (hrs) after birth with 6 milliliters (ml) of Breda virus I inoculum. These calves were necropsied at 48 hrs, 36 hrs, 95 hrs and 93.5 hrs post-inoculation (P.I.), respectively.

The two control calves GC 38 and GC 41 were inoculated orally with a

Table 1. Type of inoculum, time of commencing diarrhea, viral excretion and necropsy time of 6 gnotobiotic calves inoculated 1-2 hrs after birth

Calf number	Inoculum	Diarrhea P.I. (hrs)	Virus in feces P.I. (hrs)	Killed P.I.
GC 14	6 ml of 8×10^5 HA[a] units of Breda I	46	48	48 hrs
GC 16	6 ml of 8×10^5 HA units of Breda I	--[b]	--[c]	36 hrs
GC 18	6 ml of 8×10^5 HA units of Breda I	48	48	95 hrs
GC 20	6 ml of 8×10^5 HA units of Breda I	60	72	93.5 hrs
GC 38	Filtrate free of cryptosporidium	--	--	5 days
GC 41	Filtrate free of cryptosporidium	--	--	5 days

[a]Hemagglutination.

[b]Did not develop diarrhea.

[c]Virus was not detected in feces.

filtrate free of cryptosporidium. They were necropsied when they were 5 days old.

Inoculum

The inoculum was obtained from the cecal contents of a gnotobiotic calf GC 1 infected with Breda virus I. The contents were diluted 1:6 in Eagle's minimal essential medium, filtered through a 0.45 micron mem-

brane filter and had a final content of 8×10^5 Hemmaglutinating (HA) units of Breda virus I per ml. The final content was shown to be free of other known enteric viruses by methods previously described (Woode et al., 1982).

Experimental Procedure

The animals were intramuscularly injected with Rompun to facilitate removal from the isolator and transportation to the necropsy area. They were then individually put into deep anesthesia with sodium pentobarbital. A right flank laparotomy was performed in a length of 30 cm. Through this, the cecum was removed from the abdominal cavity aseptically to obtain sterile cecal contents for further virologic examinations. Then, one piece of the cecum was placed into 3% cold buffered glutaraldehyde, one piece was mounted onto a piece of acetate for a flat fixation in 10% neutral buffered formalin and a third piece of cecum was put in a plastic jar and immediately frozen in liquid nitrogen for further investigation by immunofluorescence. Small and large intestine were obtained as follows: a 4-7 cm loop each of duodenum, jejunum 100 cm, jejunum 320 cm, jejunum 450 cm, jejunum 700 cm behind the pyloric sphincter and the ileum 120 cm and ileum 50 cm before the ileal cecal valve were ligated and intralumenally fixed with cold 3% buffered glutaraldehyde or 10% neutral buffered formalin, respectively. At each site, a cross cut of intestine was frozen in liquid nitrogen. Two large intestinal samples were taken in the same manner at spiral colon and descending colon 20 cm proximal to the anus. From each of these intestinal sites, a flat section was

mounted on a piece of acetate before formalin fixation to avoid contraction of the intestinal wall during the fixation.

The glutaraldehyde fixed loops were each separately removed to a jar containing glutaraldehyde and after 45 min to one hr the material was processed as described below. From loops intralumenally fixed with 3% buffered glutaraldehyde, random pieces were cut with scissors. These pieces were placed on a wax plate and trimmed in fixative with a razor blade into 2 mm^2 pieces. The pieces were refixed in fresh glutaraldehyde for one hour and then washed in 0.1 M cacodylate buffer (pH 7.2) 2 times for 15 minutes. They were fixed with 1% osmium (OSO_4) in 0.1 M cacodylate buffer (pH 7.2) for 1 hr. Following fixation, specimens were rinsed in distilled water several times, dehydrated and embedded in epoxy resin. Thick sections were cut on a microtome type 4802A (LKB, Sweden) and appropriate areas were selected for thin sectioning. Thin sections were cut at 600-900 Å using a diamond knife and sections were collected on 3-mm diameter copper grids. Finally, the grids were immersed in solutions of uranyl acetate and lead citrate for staining. The HS-9 electron microscope (Hitachi, Japan) was used for electron microscopic studies.

The formalin fixed material was stored overnight in 10% neutral buffered formalin and prepared for paraffin embedding in the usual manner to obtain hematoxylin and eosin stained histologic sections.

All gut material not used for histology, electron microscopy or immunofluorescence was intralumenally fixed with formalin and saved in fixative for further examination with a dissecting stereomicroscope. Tissue

from the thymus, thyroid gland, lung, heart, spleen, rumen, reticulum, liver, omasum, abomasum, kidneys, adrenal gland, mesenteric lymph nodes and in most cases tongue, trachea and esophagus were fixed in 10% neutral buffered formalin and processed by routine paraffin techniques.

Intralumenally fixed loops were examined by a dissecting stereomicroscope after staining with methylene blue. Methylene blue was prepared at 1.4% by adding 7 gm of methylene blue powder to 500 ml of 95% alcohol; the mixture was stirred for 10 minutes and filtered. The solution was diluted with distilled water to a final concentration of 1:3.

The fixative was aspirated from each loop and methylene blue stain was injected intralumenally. The loop was gently agitated to distribute the stain in the mucosa. The stain was reaspirated and the loop was opened up gently with a scissors along the opposite side of mesenteric attachment. The opened loop was rinsed with water for 30 seconds. The tissue was placed in a glass pan and it was examined under water with a dissecting stereomicroscope. Morphological appearance of villi and villous fusion were recorded. Representative samples were taken and photographed.

The villous to crypt ratio measurements were based on a total of 20 villi and 20 crypts from 2 random pieces taken from each intralumenally 10% neutral buffered formalin fixed loop, 10 crypts and 10 villi from each piece. Each random piece of 1-square cm was cut with a scissors and it was transferred to a petri plate that contained distilled water. The piece was cut into stripes of 2 mm into a large droplet of distilled wa-

ter. A pointed forceps was used to gently remove the serosa, the connective tissue, the muscular layers and the submucosa so that villi with crypts were left. The crypt bases at the serosal side appeared transparent. The section was transferred into a methylene blue stain solution, as previously described, for 30 sec. Then, it was rinsed with distilled water. After that, the tissue was placed on a clean glass slide under a dissecting stereomicroscope. Ten rows of villi with their crypts were cut and placed separately in 10 drops of distilled water on a clean glass slide. These samples were examined by a light microscope provided with an ocular micrometer. Ten well-oriented villi and 10 straight crypts were measured from each piece. All measurements were recorded in microns and villous to crypt ratio was obtained.

Virus Isolation from Fecal and Intestinal Samples

The methods used to identify Breda virus hemagglutinin and virus particles by electron microscopy of negatively stained fecal preparations have been described previously (Woode et al., 1982).

Immunofluorescence

The indirect method was used. Acetone fixed cryostat sections of intestine were reacted with gnotobiotic calf antiserum to Breda virus I followed by rabbit anti-bovine immunoglobulin (RABIG) (Cappel Laboratories). The test was controlled by incorporating a Breda virus antibody negative serum and also by staining sections with RABIG alone.

RESULTS

Viral Excretion and Clinical Signs

Results of virus excretion and onset of diarrhea for all 6 calves are summarized in Table 1. Calf GC 16 which was killed 36 hrs P.I. did not develop diarrhea and was clinically normal until the experiment was terminated. This calf had no detectable virus in the fecal samples taken at 36 hrs when it was killed. Calf GC 14 commenced diarrhea at 46 hrs P.I. Virus excretion was detected in fecal samples 2 hrs after commencing diarrhea and it was killed at 48 hrs P.I. This calf had depression, anorexia and muscular shivering. GC 18 became diarrheic at 48 hrs P.I. when virus shedding in feces was detected. This calf was necropsied at 95 hrs P.I. GC 20 which was necropsied at 93.5 hrs P.I. developed diarrhea at 60 hrs P.I. and it shed the virus in the feces 12 hrs after diarrhea began. No clinical signs other than diarrhea were detected in GC 18 and GC 20. The two control calves (GC 38 and GC 41) remained normal until day 5 when they were necropsied.

Virus Detection

Electron microscopy and immunofluorescence were used for virus detection as summarized in Table 2. The fecal samples from the infected calves were tested for the presence of coronavirus, rotavirus, calicivirus-like agent (Newbury agent) and astrovirus. These viruses were not detected in fecal material, in cecal samples or by immunofluorescence in frozen sections from any of the calves. Breda virus I particles or viral

Table 2. Detection of Breda virus I in the intestinal mucosa of 4 inoculated calves compared to 2 control calves

		Duod	Jej 100	Jej 320	Jej 450	Jej 700	Ileum 120	Ileum 50	Cecum	Sp. colon	Desc. colon
GC 14	I.F.[a]	-[b]	-	+[c]	-	+	+	+	+	+	+
	E.M.[d]	-	-	-	-	+	+	+	-	-	+
GC 16	I.F.	-	-	-	+	-	-	-	-	-	-
	E.M.	-	-	-	+	-	-	-	-	-	-
GC 18	I.F.	-	-	-	-	+	+	+	nt[e]	+	+
	E.M.	-	-	-	-	+	+	+	-	+	+
GC 20	I.F.	-	-	-	-	+	+	+	+	+	nt
	E.M.	-	-	-	-	-	+	+	+	+	+
GC 38	I.F.	-	-	-	-	-	-	-	-	-	-
	E.M.	-	-	-	-	-	-	-	-	-	-
GC 41	I.F.	-	-	-	-	-	-	-	-	-	-
	E.M.	-	-	-	-	-	-	-	-	-	-

[a] Immunofluorescence.

[b] Negative.

[c] Positive.

[d] Electron microscopy.

[e] Not taken.

antigen were almost consistently found in all sites of the lower jejunum, the ileum and the large intestine of infected calves. Viral particles were not observed by electron microscopy in the cecum of GC 18 and samples for immunofluorescence were not taken. In GC 14, Breda virus antigens were detected by immunofluorescence in the cecum and the spiral colon but the virus particles were not observed by electron microscopy. Breda virus was also detected by either immunofluorescence or electron microscopy in 2 sites of the jejunum, jejunum 320 and jejunum 450 of GC 14 and GC 16, respectively.

Histology of the Intestine

Measurements of villous lengths and crypt depths were not made on these tissues. Subjective comparisons of villous lengths were made with the control tissues.

GC 14

Duodenum The duodenum appeared to be normal. The villi of the duodenum were irregular in shape, appeared normal in length but with a few short villi. They were covered with columnar epithelial cells. A few villi had enlarged tips with mildly dilated lacteals. Many epithelial cells were basally vacuolated in the upper 1/3 of the villi with lumenally located nuclei. The tips had a few cells with irregular shaped or round nuclei. Goblet cells were present in the basal 1/4 to 1/3 of the villi. There was moderate congestion.

The lamina propria had mild diffuse infiltration of eosinophils,

macrophages and lymphocytes. The latter 2 types of cells will be considered mononuclear cells wherever they are mentioned. There were moderate multifocal dilated lymphatics in the lamina propria and submucosa. Crypts were stained darkly and contained many goblet cells. There were 5 crypt abscesses (Table 3). These crypts had low columnar to cuboidal or flat epithelium with pale round to ovoid nuclei. The lumens of these crypts were filled with homogenous or granular acidophilic material and partly degenerated eosinophils. There was one dome structure. Mitotic figures were 1.2/crypt (Table 3).

Jejunum In the jejunum, there was mild congestion. The villi were normal in length and were covered by columnar epithelial cells that were basally vacuolated in the upper 1/2 with lumenally located nuclei. Moderate numbers of tips in jejunum 100 and 320 had apical cells with irregular or round nuclei. High numbers of karyorrhectic cells were observed at the base of jejunum 700 villi (Table 4). The villi in general were irregular and thickened in jejunum 700. There was mild villous lacteal dilation in all sites. Goblet cells were present in the basal 1/4 to 1/2 of the villi. In all sections except in the jejunum 320, some villi were branched into 2 villi from their bases and appeared to be split from 1 base.

The lamina propria had mild diffuse infiltration of eosinophils and mononuclear cells. The degree of lymphatic dilation in the lamina propria and submucosa varied from moderate to severe and from multifocal to diffuse without detectable regularity. The lamina propria of jejunum 700 had

Table 3. Numbers of crypt abscesses and average numbers of mitotic figures in 10 crypts in 4 infected and 2 control calves

		Duod	Jej 100	Jej 320	Jej 450	Jej 700	Ileum 120	Ileum 50	Cecum	Sp. colon	Desc. colon
GC 14	Crypt abscesses	5	4	1	1	8	-[a]	-	-	-	-
	Mitotic figures[b]	1.2	1.5	2.2	1.85	.9	1.7	1.1	.6	1.6	1.6
GC 16	Crypt abscesses	1	-	1	-	-	-	-	-	-	-
	Mitotic figures	1.2	.7	1.2	.8	1.5	1.7	2.1	1.7	1.7	1.1
GC 18	Crypt abscesses	5	9	6	10+[c]	10+	4	-	-	-	-
	Mitotic figures	3.2	2.5	3.1	4.2	3	2.8	3.5	.6	1.4	2.9
GC 20	Crypt abscesses	2	-	-	3	10+	2	-	-	-	-
	Mitotic figures	4.4	3.4	4.4	5.9	2.6	3.3	2.7	3.4	3.2	3.4

GC 38	Crypt abscesses	2	1	-	-	1	2	1	-	-	-
	Mitotic figures	2.4	1.7	1.8	1.8	1.7	1.5	1.5	.9	1.2	1.5
GC 41	Crypt abscesses	5	2	5	2	1	-	-	-	-	-
	Mitotic figures	2.5	3.1	3.1	3.1	3.7	2	2.5	1.1	.5	.8

[a]No crypt abscess detected.

[b]Average of mitotic figures in 10 well-oriented crypts.

[c]More than 10 crypt abscesses present.

Table 4. Estimated numbers of necrotic cells in bases of villi and crypts, and numbers of macrophages containing cellular debris present in lamina propria of 4 calves infected with Breda virus I compared to 2 control calves

		Duod	Jej 100	Jej 320	Jej 450	Jej 700	Ileum 120	Ileum 50	Cecum	Sp. colon	Desc. colon
	Villous bases	-[a]	-	-	-	+++	++	++	-	-	-
GC 14	Crypts	-	-	-	-	++	++	++	-	-	-
	Lamina propria	-	-	-	-	+++	+++	+++	-	-	-
	Villous bases	-	-	-	-	-	-	-	-	-	-
GC 16	Crypts	-	-	-	-	-	-	-	-	-	-
	Lamina propria	-	-	-	-	-	-	-	-	-	-
	Villous bases	-	-	-	++	++	++	++	-	-	-
GC 18	Crypts	-	-	-	++	+++	++	++	+	++	++
	Lamina propria	-	-	-	+++	+++	+++	+++	-	+++	+++
	Villous bases	-	-	-	-	+++	+	+	-	-	
GC 20	Crypts	-	-	-	-	+++	++	+	+	++	+++
	Lamina propria	-	-	-	-	+++	++	+	-	+	+++

	Villous bases	-	-	-	-	-	-	-	-	-	-
GC 38	Crypts	-	-	-	-	-	-	-	-	-	-
	Lamina propria	-	-	-	-	-	-	-	-	-	-
	Villous bases	-	-	-	-	-	-	-	-	-	-
GC 41	Crypts	-	-	-	-	-	-	-	-	-	-
	Lamina propria	-	-	-	-	-	-	-	-	-	-

[a] - = no change was detected.
+ = a few cells detected.
++ = moderate numbers of cells detected.
+++ = high numbers of cells detected.

high numbers of macrophages containing cellular debris (Table 4).

The crypts of all jejunal sections were stained darkly and contained goblet cells. There were several crypt abscesses (Table 3) as described at the duodenal site. Two crypt abscesses with pericryptal infiltration of eosinophils and macrophages were observed in jejunum 320 and 700. Moderate numbers of crypt epithelial cells were karyorrhectic in jejunum 700 (Table 4). There were 2 areas with dome structures in jejunum 320 and 450, with lymphocyte depletion in central areas. Mitotic figures were 1.5, 2.2, 1.85 and 0.9/crypt in jejunum 100, 320, 450 and 700, respectively (Table 3).

Ileum Moderate to severe general congestion was observed in the ileum. The villi were irregular in shape, short and stunted with some mild to moderate dilation of lacteals in the apical portions. The villi were covered by immature epithelial cells (Table 5). These cells were cuboidal with large, pale, round, centrally located nuclei. Some cells with lumenally located vacuoles were present. Flat epithelial cells were observed on short, stunted villi. However, the iluem 120 had columnar epithelial cells in the basal 1/4 of the villi. The immature cells were dissociated and sloughed in some areas. There were many completely denuded villi; this change resulted in exposing the villous lamina propria to the lumen in both ileal sites (Table 5). In ileum 50, several villi were necrotic resulting in loss of villous architecture with sloughed epithelial cells, inflammatory cells and necrotic cellular debris in the lumen (Figures 1 and 2). Moderate numbers of cells at the villous bases of both sites were necrotic (Table 4). There were some fusions at vil-

Table 5. Presence of immature and necrotic cells and epithelial fusion in 4 infected calves compared to 2 control calves

	Duod	Jej 100	Jej 320	Jej 450	Jej 700	Ileum 120	Ileum 50	Cecum	Sp. colon	Desc. colon
GC 14	-[a]	-	-	-	-	+[b]	+	+	+	+
	-	-	-	-	-	*[c]	*	-	*	*
					-	x[d]	-	-	x	x
GC 16	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
GC 18	-	-	-	-	+	+	+	-	+	+
					-	-	-	-	*	-
	-	-	-	-	x	x	x	-	-	-
GC 20	-	-	-	-	-	+	+	+	+	+
	-	-	-	-	-	*	-	-	-	*
	-	-	-	-	-	x	x	x	x	-
GC 38	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-
GC 41	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-

[a] No detectable change.

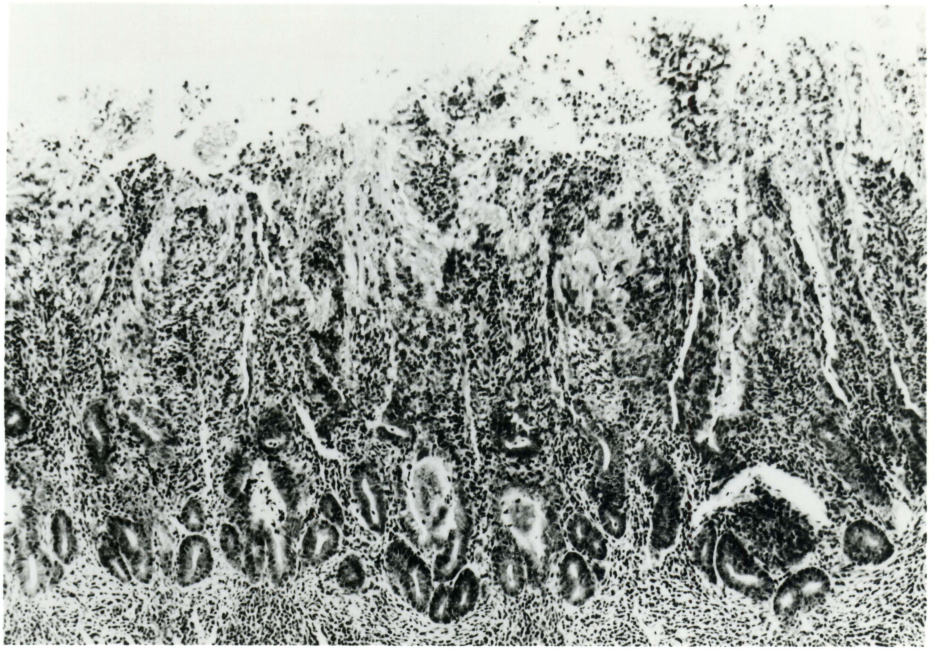
[b] Presence of immature epithelial cells.

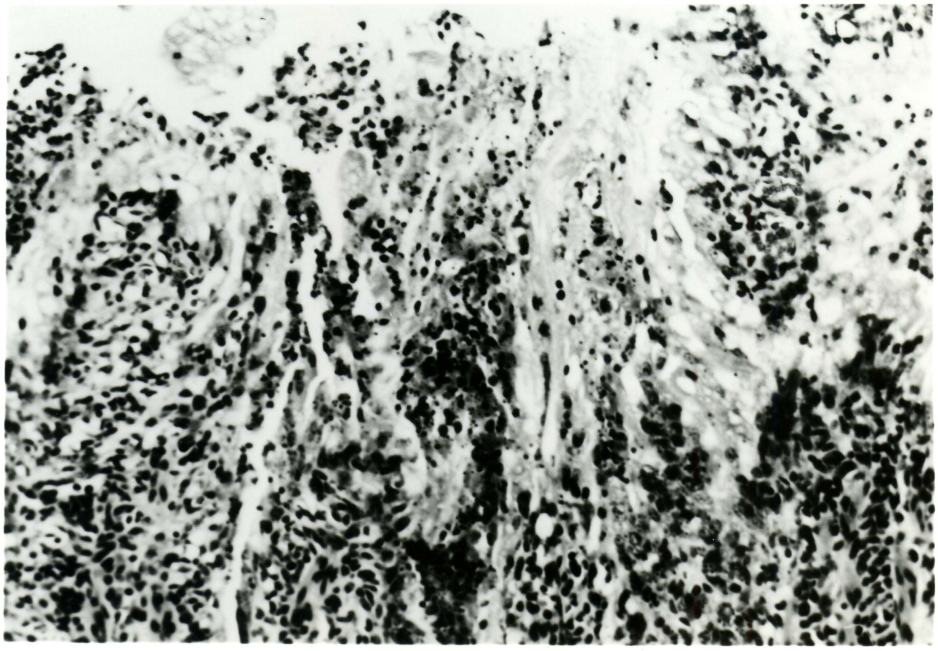
[c] Presence of necrotic cells.

[d] Villous fusion and fusion of colonic folds.

Figure 1. Ileum 50 of GC 14 (48 hrs P.I.): severe necrosis of villi, cellular debris in the lumen, loss of villous architecture [for comparison see Figures 11 and 12] (75x)

Figure 2. Ileum 50 of GC 14 (48 hrs P.I.): sloughing of epithelial cells with necrotic cellular debris in the lumen and inflammatory cells in the lumen and lamina propria [for comparison see Figures 11 and 12] (420x)





lous tips in the ileum 120 (Table 5). Goblet cells were not detected on the villi.

The lamina propria had mild to moderate infiltration of eosinophils and high numbers of mononuclear cells. High numbers of macrophages containing cellular debris were observed in the lamina propria of ileum 120 and 50 (Table 4).

The crypt lining cells were darkly stained and the crypt lumens were moderately dilated. The crypts contained a few goblet cells. Moderate numbers of crypt epithelial cells were karyorrhectic (Table 4). Peyer's patches had moderate lymphocyte depletion in the central areas and 1 dome had vacuolated and dissociated epithelial cells on the tip. Mitotic figures were 1.7 and 1.1/crypt in the ileum 120 and 50, respectively (Table 3).

Large intestine The surface mucosal folds of the large intestine (cecum, spiral, colon and descending colon) were covered with columnar epithelial cells with abundant goblet cells. However, there were some low columnar or cuboidal epithelial cells detectable. Few patchy fold fusions and sloughed epithelial cells were observed in the 2 sites of the colon (Table 5). There was moderate congestion.

Moderate focal to multifocal lymphatic dilation was noticed in the lamina propria and submucosa. Few scattered eosinophils and moderate infiltration of mononuclear cells were seen in the lamina propria. Crypts were darkly stained with mild multifocal to diffuse dilation except in the descending colon. Mitotic figures were 0.6, 1.6 and 1.6/crypt in the cecum, the spiral colon and the descending colon, respectively (Table 3).

GC 16

Duodenum The duodenum appeared normal. The villi of the duodenum were irregular in shape, normal in length and covered by columnar epithelial cells. Some villi had enlarged tips that had moderately dilated lacteals. Goblet cells were present in the basal 1/2 of the villi. There was general mild congestion. One branching villous was observed.

The lamina propria had mild to moderate infiltration of mononuclear cells and eosinophils. There was moderate to severe multifocal lymphatic dilation in the lamina propria and submucosa. The crypts were stained darkly. One crypt abscess was present (Table 3). The duodenal section had 2 dome structures and 2 lymphocyte aggregations in the submucosal area. The mitotic figures were 1.2/crypt (Table 3).

Jejunum The jejunum appeared normal. The jejunum was mildly congested and the villi were irregular in shape and normal in length. A few mildly dilated lacteals were observed. The villi were covered by columnar epithelial cells which were basally vacuolated in the upper 1/2 of the villi. Goblet cells were observed in the basal 1/4 to 1/2 of the villi. A few branching villi were detected in all sites except in jejunum 700. This site had a few short villi. A few cells with round nuclei were seen in a few villous tips of jejunum 100 and 320.

There was moderate cellularity of the lamina propria consisting of mononuclear cells and eosinophils. The lymphatics were dilated to varying degrees in the lamina propria and submucosa. The crypts were stained darkly and contained goblet cells. A few crypts were mildly dilated in

the jejunum 450. One crypt abscess was detected in jejunum 320 (Table 3). Two large areas of dome structures were observed in the jejunum 100 and 320. Mitotic figures were 0.7, 1.2, 0.8 and 1.5/crypt in jejunum 100, 320, 450 and 700, respectively (Table 3).

Ileum The ileum appeared normal. The villi were irregular in shape and normal in length. Some short villi were present in the ileum 120. There were mild to moderately dilated lacteals. The ileum had general mild congestion. The epithelial cells covering the villi were columnar and they were vacuolated lumenally in the upper 2/3 of the villi. Goblet cells were located in the basal 1/4 of the villi. One branching villous was seen in each site of the ileum.

The lamina propria was infiltrated with moderate numbers of eosinophils and mononuclear cells. The lymphatics were dilated to varying degrees in the lamina propria and submucosa. The crypts were stained darkly and had goblet cells. Mitotic figures were 1.7 and 2.1/crypt in the ileum 120 and 50, respectively (Table 3). Many Peyer's patches were markedly depleted in the centers. The apical cells of the domes were rounded.

Large intestine The large intestine appeared to be normal. The surface mucosa was covered by columnar epithelial cells with abundant goblet cells. There was moderate general congestion. The lamina propria had a few eosinophils and moderate numbers of mononuclear cells. There was mild dilation of the lamina propria and submucosal lymphatics. Crypts were stained darkly and moderate multifocal crypt dilation was observed. Mitotic figures were 1.7, 1.7 and 1.1 in the cecum, spiral colon and de-

scending colon, respectively (Table 3).

GC 18

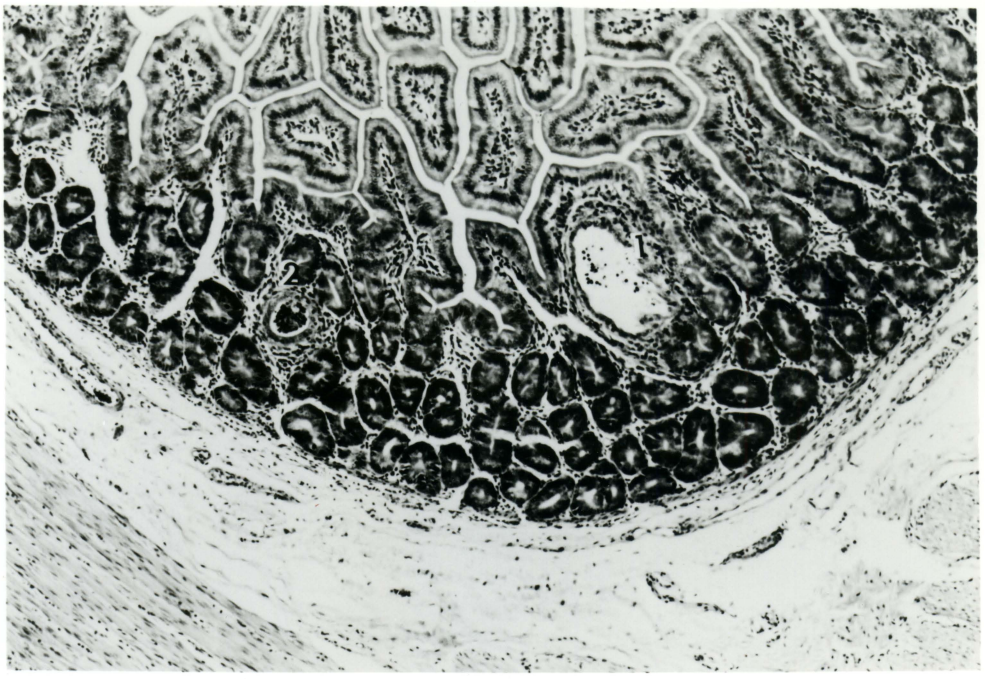
Duodenum The duodenum appeared to be normal. The villi of the duodenum were normal in length and furrowed in the upper 1/3. There were a few mildly dilated lacteals in the enlarged tips or in the mid-villous. The villi were covered by columnar epithelial cells. Some villi had vacuolated epithelial cells in the upper 1/4. These vacuoles were in the cell bases and the nuclei were located to the lumenal side. Some tips of villi had rounded nucleated cells. Goblet cells were observed in the basal 1/2 of the villi. The duodenum had general mild congestion.

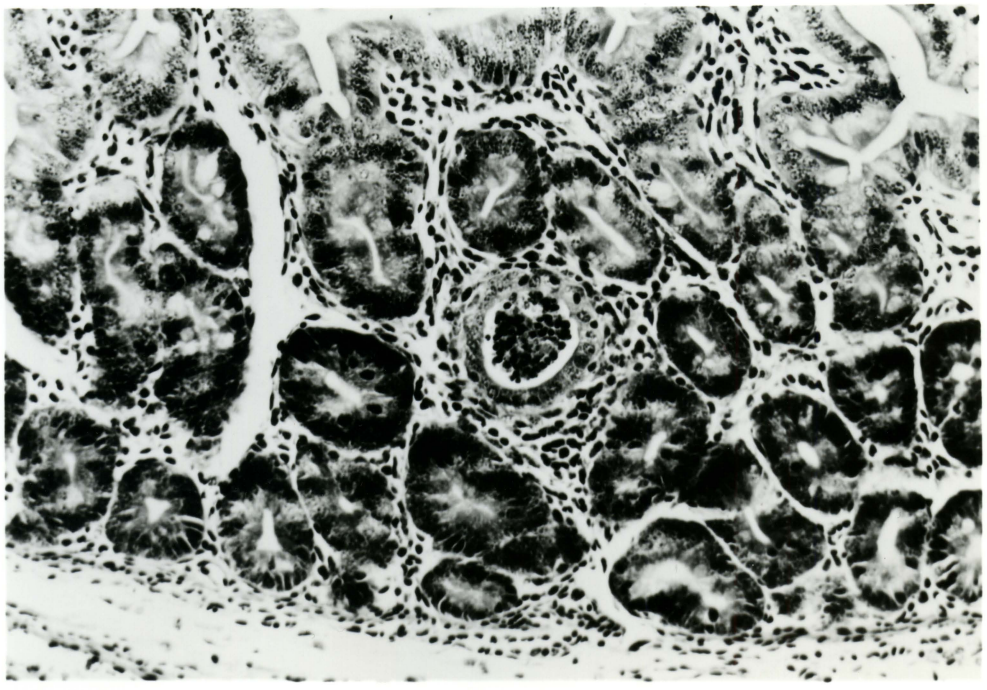
The lamina propria had severe diffuse infiltration of eosinophils and moderate numbers of mononuclear cells. There was moderate multifocal lymphatic dilation. The crypts were stained darkly and they contained goblet cells. There were 5 crypt abscesses scattered in this site (Table 3, Figures 3 and 4). One crypt abscess had pericryptal eosinophils, macrophages and a few lymphocytes. Mitotic figures were 3.2/crypt (Table 3).

Jejunum The jejunum had mild to moderate congestion and dilated lacteals. The villi were normal in length except jejunum 700 which had several short villi. The villi were furrowed in the upper 1/3 to 1/2 except in jejunum 700 where the villi were irregular in shape. The villi were covered by columnar epithelial cells which were basally vacuolated in the upper 1/4 to 1/2 of most of the villi. Most of the upper 1/2 of the villi in jejunum 700 were covered by cuboidal epithelial cells which

Figure 3. Duodenum of GC 18 (95 hrs P.I.): 2 crypt abscesses: 1) dilated crypts with flat epithelial cells and lumenal inflammatory cells, 2) crypt abscess with pericryptal inflammatory cells and lumenal acidophilic material and inflammatory cells (85x)

Figure 4. Duodenum of GC 18 (95 hrs P.I.): crypt abscess with pericryptal inflammatory cells and lumenal acidophilic material and inflammatory cells (220x)


1
2



had round, pale, large nuclei and vacuolated cytoplasm (Table 5). Moderate numbers of karyorrhectic epithelial cells were detected in villous bases of jejunum 450 and 700 (Table 4). Fused villi were observed at jejunum 700 (Table 5).

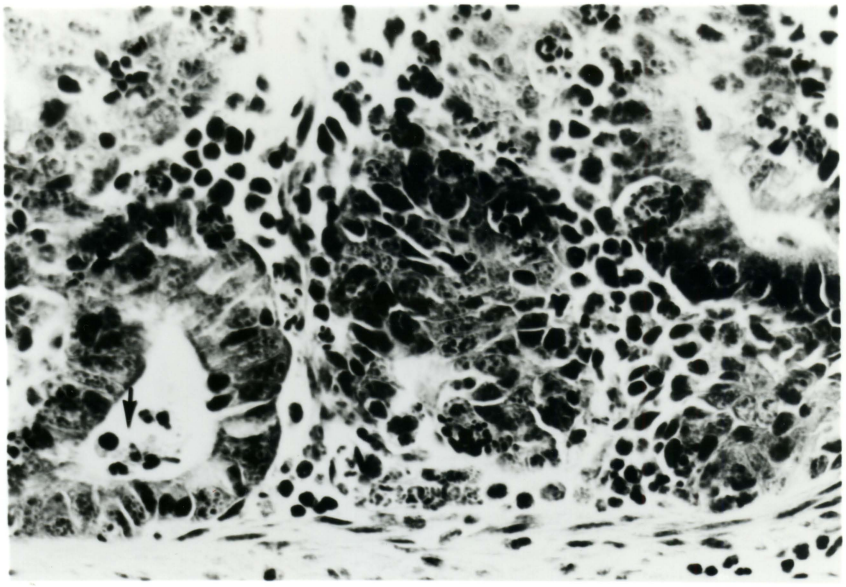
There were varying degrees of lymphatic dilation in the lamina propria and submucosa. The lamina propria had moderate to severe diffuse infiltration of mononuclear cells and eosinophils. High numbers of macrophages containing cellular debris were observed in the lamina propria of jejunum 450 and 700 (Table 4). Crypts were stained darkly. Many crypt abscesses were found in all 4 sites (Table 3). In jejunum 100 and 320, a few crypt abscesses had pericryptal cells consisting of eosinophils, macrophages and lymphocytes. Varying numbers of crypt cells were karyorrhectic in jejunum 450 and 700 (Table 4, Figure 5). They also had mild multifocal crypt dilation. Mitotic figures were 2.5, 3.1, 4.2 and 3.0/crypt in the jejunum 100, 320, 450 and 700, respectively (Table 3).

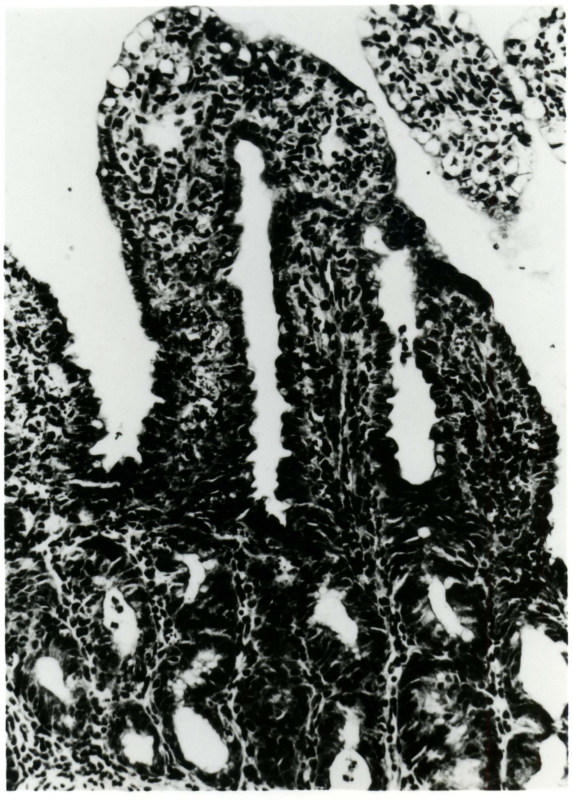
Ileum The villi were irregular in shape and short. The villi were covered by columnar epithelial cells in the basal 1/2. The other halves were covered by cuboidal epithelial cells that had round, large, pale nuclei with cytoplasmic irregular vacuoles (Table 5). Some of these nuclei were irregular in the ileum 50. There were moderate numbers of karyorrhectic cells in the villous bases of both ileal sites (Table 4). Some tips were fused (Table 5, Figure 6). Goblet cells were present in the basal 1/4 to 1/3 of the villi. There was moderate general congestion and lacteal dilation.

The lamina propria had moderate to severe diffuse infiltration of

Figure 5. Jejunum 700 of GC 18 (95 hrs P.I.): crypt epithelial necrosis. Inflammatory cells in crypt lumen [arrow] (422x)

Figure 6. Ileum 50 of GC 18 (95 hrs P.I.): fusion of 3 tips of villi [for comparison see Figures 11 and 12] (178x)





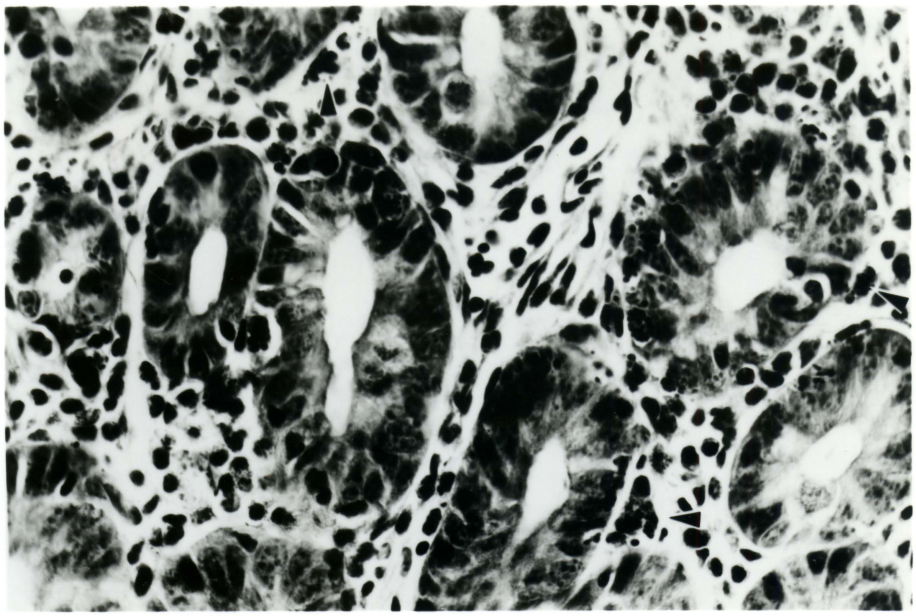
mononuclear cells and eosinophils with high numbers of macrophages containing cellular debris in both sites (Table 4, Figure 7). The crypts were stained darkly. There were 4 crypt abscesses present in the ileum 120 (Table 3). There was moderate multifocal to diffuse crypt dilation in the ileum 120 and ileum 50, respectively. Moderate numbers of karyorrhectic crypt epithelial cells were observed (Table 4). The M cells in the apical dome surface of the ileum 120 had large pale to ovoid nuclei with irregular cell surface. The Peyer's patches in both sites had undergone different degrees of central lymphocyte depletion. Mitotic figures were 2.8 and 3.5/crypt in the ileum 120 and 50, respectively (Table 3). The lymphatics were dilated in the submucosa.

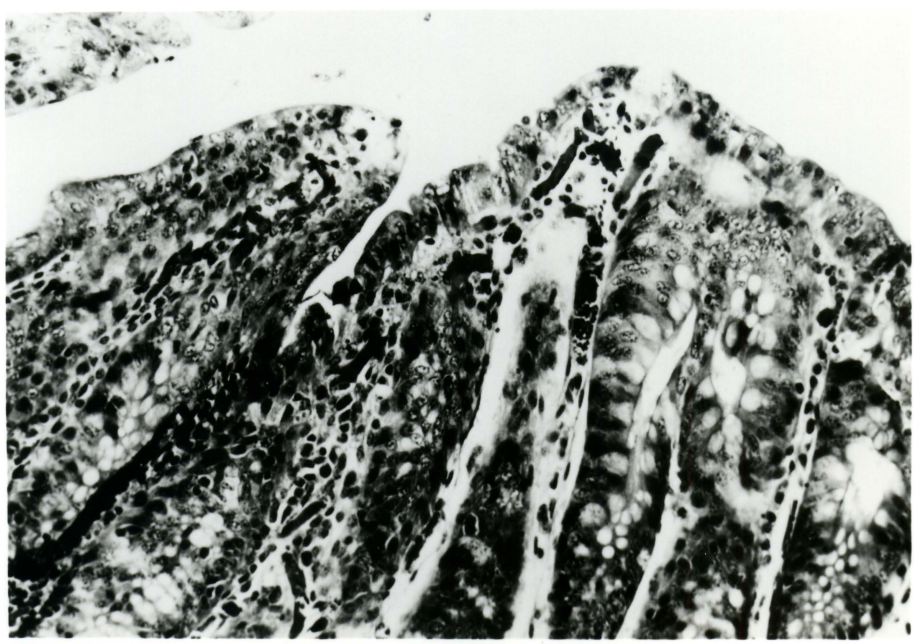
Large intestine The surface mucosa of the large intestine was covered with columnar epithelial cells with the presence of abundant numbers of goblet cells. There were no detectable colonic folds in some areas where the epithelial cells became cuboidal with round or irregular nuclei (Table 5). Some of these cells in the spiral colon became vacuolated and had karyolytic nuclei. These cells were detached and sloughed in some areas (Figure 8). There was moderate to severe general congestion.

There was mild multifocal lymphatic dilation in the lamina propria with a few scattered eosinophils and moderate numbers of mononuclear cells. The lamina propria of the colon had high numbers of macrophages containing cellular debris (Table 4). The crypts were darkly stained and mild to moderate crypt dilation was observed. Karyorrhectic epithelial

Figure 7. Ileum 120 of GC 18 (95 hrs P.I.): macrophages containing cellular debris in the lamina propria [arrowhead] (456x)

Figure 8. Spiral colon of GC 18 (95 hrs P.I.): necrotic epithelial cells on the mucosal surface (178x)





cells of crypts were scattered in all 3 sites; however, there were very few in the cecum (Table 4). The necrotic cells occasionally were located close to the crypt orifices. Mitotic figures were 0.6, 1.4 and 2.9 in the cecum, spiral colon and descending colon, respectively (Table 3). Some lymphatics were dilated in the submucosa.

GC 20

Duodenum The duodenum appeared to be normal. The villi of the duodenum were normal in length. The villi were irregular in shape and had moderately dilated lacteals. A few villi had enlarged tips. Columnar epithelial cells covered the villi. Some tips had cells with irregular or round nuclei. There was no cell vacuolation of the villi. Goblet cells were present in the basal 1/3 to 1/2 of the villi. There was moderate to severe congestion.

There was mild diffuse lymphatic dilation in the submucosa and in the lamina propria which also had severe diffuse infiltration of eosinophils and moderate numbers of mononuclear cells. The crypts were stained darkly and contained goblet cells. There were 2 crypt abscesses (Table 3). The mitotic figures were 4.4/crypt (Table 3). Three dome structures were observed.

Jejunum The villi in all sites of the jejunum were irregular in shape and normal in length; however, the villi were shorter in jejunum 700 compared to the other three sites. The villous lacteals were moderately dilated. The villi were covered by columnar epithelial cells. At their apex, some villi had cells with irregular nuclei. There was no

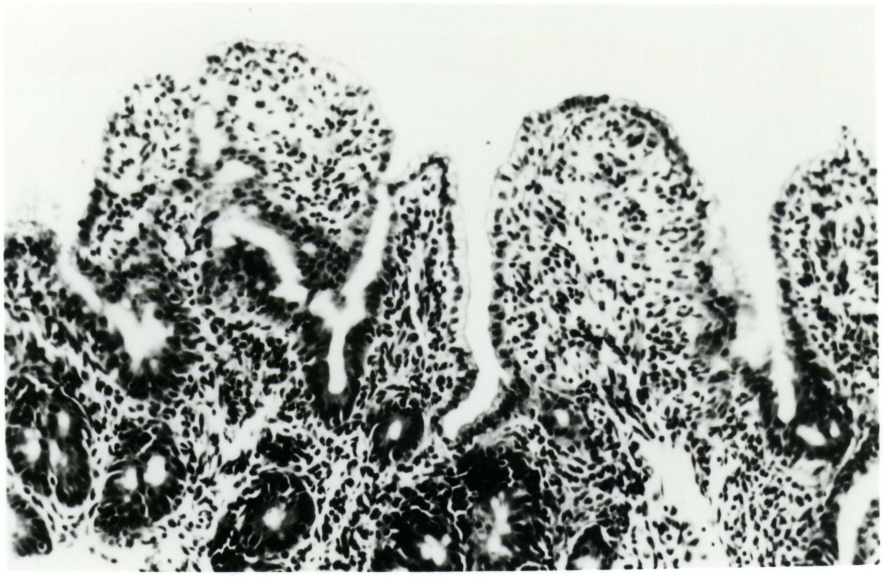
vacuolation of the epithelial cells except a few cells that were basally vacuolated in villous tips of jejunum 450. High numbers of karyorrhectic epithelial cells were present in the villous bases of jejunum 700 (Table 4). Goblet cells were present in the basal 1/4 of the villi. There was moderate general congestion.

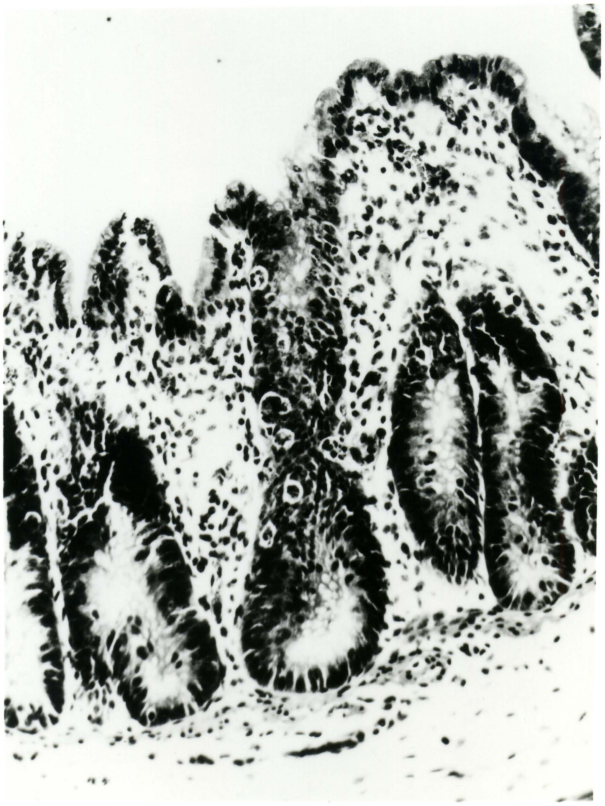
The lamina propria had severe infiltration of eosinophils and mononuclear cells except in the jejunum 100. Varying degrees of lymphatic dilation were observed. High numbers of macrophages containing cellular debris were observed in the lamina propria of the jejunum 700 (Table 4). Crypts were stained darkly and some were moderately dilated in the jejunum 700. Several crypt abscesses were observed in the jejunum 450 and 700 (Table 3). High numbers of crypt epithelial cells were karyorrhectic in the jejunum 700 (Table 4). Mitotic figures were 3.4, 4.4, 5.9 and 2.6 per crypt in the jejunum 100, 320, 450 and 700, respectively (Table 3).

Ileum The villi were irregular, short and consistently stunted in the ileum 50 (Figure 9). In the ileum 120, there were a few mildly dilated lacteals. The epithelial cells in the upper 1/4 to 1/3 were immature and were characterized by large round nuclei and lumenal standing vacuoles (Table 5). Some short stunted villi were completely covered by cuboidal epithelial cells. Occasionally, columnar epithelial cells covered some villi and sometimes they were restricted to the basal 3/4 or 2/3 of the villi. Few tips in the ileum 120 had sloughed epithelial cells (Table 5). Few karyorrhectic cells were present in the villous bases of both sites (Table 4). Fusion of villous tips and villous sides

Figure 9. Ileum 50 of GC 20 (93.5 hrs P.I.): short, stunted villi with fusion on tips of villi (183x) [for comparison see Figures 11 and 12]

Figure 10. Spiral colon of GC 20 (93.5 hrs P.I.): necrotic epithelial cells of crypts which were occasionally observed at crypt orifices (173x)





was observed (Table 5) and was more severe in the ileum 50. Goblet cells were present in the basal portions of some villi. There was mild to moderate general congestion.

There was mild multifocal to diffuse lymphatic dilation in the lamina propria and the submucosa. The lamina propria was diffusely infiltrated with high numbers of eosinophils and moderate numbers of mononuclear cells. Macrophages containing cellular debris were scattered throughout the mucosal parenchyma of both sites (Table 4). Crypts were stained darkly with mild to moderate diffuse dilation. Goblet cells were present in some crypts. Karyorrhectic cryptal epithelial cells were present in both sites (Table 4). Two crypt abscesses were observed in the ileum 120 (Table 3). The Peyer's patches had moderate to severe central lymphoid depletion. M cells on dome tips of both sites were vacuolated and had round, large nuclei. One dome showed a few sloughed epithelial cells. Mitotic figures were 3.3 and 2.7/crypt in the ileum 120 and 50, respectively (Table 3).

Large intestine The surface mucosa of the large intestine was covered with columnar epithelial cells and abundant goblet cells. There were moderate to severe multifocal areas of low columnar to cuboidal epithelial cells which had large pale round nuclei and vacuolated cytoplasm. Some of these nuclei were pyknotic. This change was less severe in the cecum. Immature cells were sloughed in many areas of the descending colon. Besides there were many karyorrhectic epithelial cells between the basal colonic folds and crypt orifices. Few patchy fusions of the

folds were present in the cecum and in the spiral colon (Table 5). There was moderate to severe general congestion.

There was mild to moderate multifocal dilation of lymphatics in the lamina propria and the submucosa with mild diffuse infiltration of eosinophils and moderate numbers of mononuclear cells. There were more macrophages containing cellular debris of the descending colon than in the spiral colon (Table 4). The crypts were darkly stained with abundant goblet cells. There was a varying degree of crypt dilation in the colon. Karyorrhectic crypt epithelial cells were observed in all 3 sections (Table 4, Figure 10). These cells were less in the cecum and more prominent in the descending colon. Mitotic figures were 3.4, 3.2 and 3.4/crypt in the cecum, spiral colon and descending colon, respectively (Table 3).

GC 38

Duodenum The villi were tall and straight in the upper 1/4 and covered by columnar epithelial cells. Some villi had mildly dilated lacteals. Goblet cells were present in the basal 1/2 of the villi. There was general mild congestion. The lamina propria was infiltrated with mild to moderate numbers of eosinophils and mononuclear cells. A few mildly dilated lymphatics were observed in the lamina propria and submucosa. The crypts were stained darkly and contained goblet cells. There were 2 crypt abscesses (Table 3). Mitotic figures were 2.4/crypt (Table 3). The duodenum had one large area of dome structures.

Jejunum The villi were tall and straight with wide furrows. However, the villi in jejunum 700 became thick and the upper 1/3 of some

villi were irregular. Columnar epithelial cells covered the villi, some of which had moderately dilated lacteals. The epithelial cells in the upper 1/4 to 1/2 of the jejunum 450 and 700 were lumenally vacuolated. Epithelial cells of a few tips in the jejunum 320 had some vacuoles only. Goblet cells were present in the basal 1/3 to 1/2 of the villi. There was moderate general congestion.

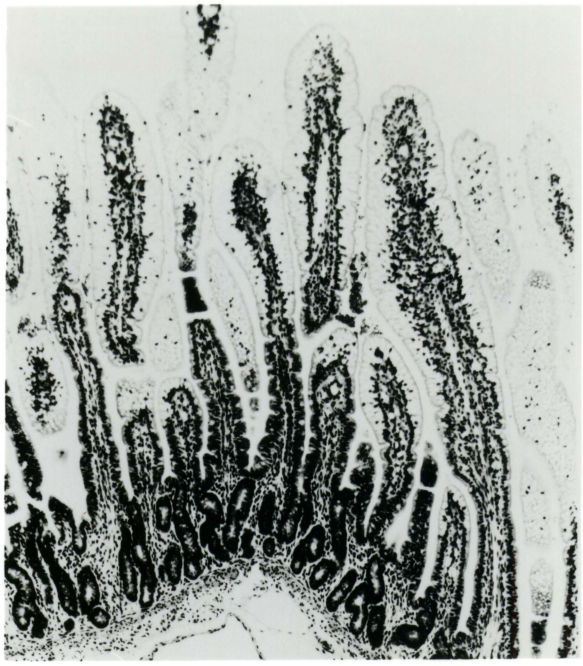
The lamina propria was infiltrated with moderate numbers of eosinophils and mononuclear cells. There were moderate multifocal dilated lymphatics in the lamina propria and submucosa. Crypts were darkly stained and they contained goblet cells. There was one crypt abscess in each of jejunum 100 and 700 with pericryptal eosinophils and macrophages (Table 3). Mitotic figures were 1.7, 1.8, 1.8 and 1.7 in the jejunum 100, 320, 450 and 700, respectively (Table 3). There was one large area of dome structures in the jejunum 450.

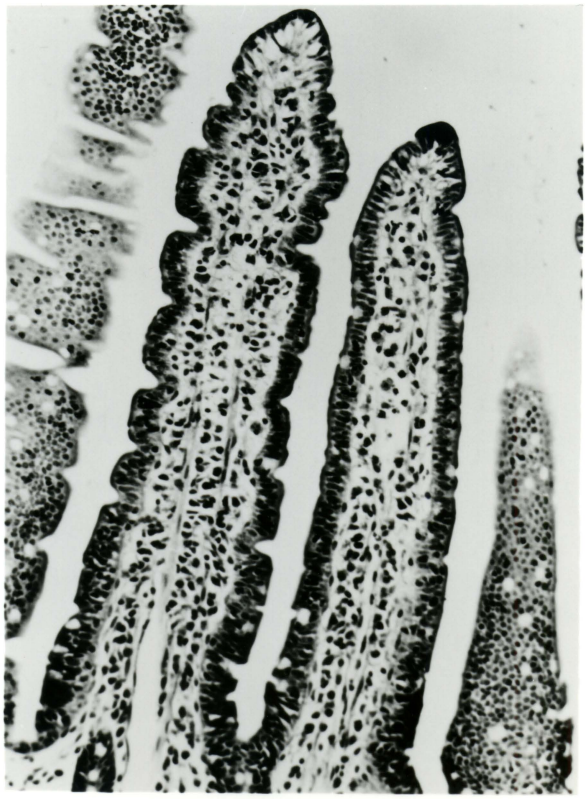
Ileum The villi were tall and covered by columnar epithelial cells. Irregularity of the villi was observed in the upper 1/2 in the ileum 50 with some enlarged tips. The villous epithelial cells generally were lumenally vacuolated in the upper 1/2 of the villi which also had some mildly dilated lacteals (Figure 11). Goblet cells were present in the basal 1/2 of the villi. There was mild general congestion.

The lamina propria was infiltrated with moderate numbers of eosinophils and mononuclear cells. There were also multifocal mildly dilated lymphatics. Crypts were stained darkly and goblet cells were present. Three crypt abscesses were present in the ileum (Table 3). A few crypts

Figure 11. Ileum 50 of GC 38 (control): tall, straight villi covered by columnar epithelial cells which were lumenally vacuolated (70x)

Figure 12. Ileum 120 of GC 41 (control, 5 days old): straight, tall villi covered with columnar epithelial cells (188x)





were mildly dilated. Mitotic figures were 1.5/crypt in each site (Table 3). Peyer's patches had mild lymphocyte depletion in the centers. The M cells were rounded. Two domes in ileum 50 had detached epithelial cells, a few of which sloughed into the lumen.

Large intestine The surface mucosa was covered by columnar epithelial cells and abundant goblet cells. General mild congestion was observed in the colon. The lamina propria of the large intestine was infiltrated with moderate numbers of mononuclear cells and a few eosinophils. The crypts were stained darkly and had abundant numbers of goblet cells. There was mild to moderate multifocal crypt dilation. Mitotic figures were 0.9, 1.2 and 1.5 in the cecum, spiral colon and descending colon, respectively (Table 3).

GC 41

Duodenum The villi were straight with wide furrows, moderate in length and covered with columnar epithelial cells. There was no cellular cytoplasmic vacuolation. There were moderately dilated lacteals. Goblet cells were present in the basal 1/3 to 1/2 of the villi. There was general moderate congestion.

There was mild cellularity of the lamina propria, consisting of eosinophils and mononuclear cells. Moderate multifocal lymphatic dilation was also observed with mild edema. Two areas of lymphocyte aggregation were observed in the lamina propria. Crypts were darkly stained and had many goblet cells. There were 5 crypt abscesses (Table 3). Two crypts

had pericryptal infiltration of eosinophils and macrophages. Mitotic figures were 2.5/crypt (Table 3).

Jejunum The villi were tall and covered by columnar epithelial cells. The villi of the jejunum 700 appeared thickened compared to other parts of the jejunum. Generally, the villi tended to be straight in the basal 2/3 to 3/4. The lacteals were dilated to varying degrees. The epithelial cells of some apical villi were vacuolated lumenally in the jejunum 100 and 320, basally in the 450 and lumenally and basally in the 700. Goblet cells were present in the basal 2/3 to 3/4 of the villi. One branching villous was detected in each jejunum 100 and 320. There was general mild congestion.

The lamina propria was infiltrated with mild numbers of eosinophils and moderate numbers of mononuclear cells. The lymphatics of the lamina propria and submucosa were dilated to varying degrees with mild edema in all sites. The crypts were lightly basophilic with the presence of many goblet cells. Several crypt abscesses were detected in all sites of the jejunum (Table 3). A few of these crypts were surrounded by eosinophils, macrophages and lymphocytes. There were multifocal mildly dilated crypts in the jejunum 450 and 700. Mitotic figures were 3.1, 3.1, 3.1 and 3.7 in the jejunum 100, 320, 450 and 700, respectively (Table 3).

Ileum The villi were regular, straight and moderate in length with some short villi. However, some irregular villi were present. Few mildly dilated lacteals were observed. Columnar epithelial cells covered the villi (Figure 12), some of which had apical lumenal vacuolated epi-

thelial cells. Goblet cells were present in the basal 1/2 to 3/4 of the villi. Ileum 50 had 3 branching villi. There was general mild to moderate congestion.

The lamina propria was infiltrated with mild to moderate numbers of eosinophils and mononuclear cells. There were various degrees of lymphatic dilation in the lamina propria. The submucosa of the ileum 50 was edematous and it had dilated lymphatics and blood vessels. Peyer's patches had moderate depletion of lymphocytes in the centers. Goblet cells were present in the crypts. Mitotic figures were 2.0 and 2.5/crypt in the ileum 120 and 50, respectively (Table 3).

Large intestine The surface mucosa was covered with columnar epithelial cells with an abundance of goblet cells. There was moderate general congestion.

The lamina propria was infiltrated with moderate numbers of mononuclear cells and few eosinophils. The spiral colon had submucosal dilated lymphatics. The crypts were stained lightly basophilic and had abundant numbers of goblet cells. Multifocal mild to moderately dilated crypts were present. Mitotic figures were 1.1, 0.5 and 0.8/crypt in the cecum, spiral colon and descending colon, respectively (Table 3).

The Methylene Blue Procedure

The intestinal tissue of GC 14 was not evaluated by methylene blue stain because this technique was applied later. Other infected and control calves were examined in a similar manner.

GC 16

The villi of the duodenum were moderate in length and uniform in shape. However, moderate numbers of short and tall villi were present. The villi were either flat or cylindrical (finger-shaped) and the tips were generally rounded. Some tips were pointed and others were enlarged.

In the jejunum, villi were moderate in length with either a flat tongue shape or cylindrical shape. Moderate numbers of short and tall villi were present in the terminal portion of the jejunum. Englarged and pointed tips were occasionally observed.

The ileal villi were shorter than those in the jejunum and were mildly stunted. The villi were flat with rounded tips and some tongue-shaped villi were present.

GC 18

The villi of the duodenum were tall to moderate in length and flat in shape with rounded tips. Generally, the villi were stained uniformly except the distal portion of their bases. The tips had small white unstained dot-like areas.

The villi in the jejunum were tall and either flat or cylindrical with rounded tips. However, there were many moderate to short villi in the distal segment of the jejunum. These villi were stained in the upper 1/3 unlike the villi in the upper sites of the jejunum where the villi were stained more intensely all over the surface. Some tongue-shaped villi were seen in the jejunum 100 to 320. Several branching villi were detected in the jejunum 100.

The villi in the ileum were short, stunted or plump with rounded tips although moderate numbers of tall, cylindrical villi were present. The latter were stained darkly and uniformly. High numbers of the short, plump villi were fused in groups of 2-5 villi. These villi were palely stained. These types of villi were more predominant in the last segment of the ileum.

GC 20

The villi in the duodenum were moderate in length and flat or cylindrical with rounded tips. However, a few short, plump villi were observed. Pointed and enlarged tips were seen on some villi. Generally, the villi were well-stained except the basal portions.

In the jejunum, the villi were tall to moderate in length and flat or cylindrical with rounded tips. All segments had scattered short villi, some of which had pointed tips and were stained in the upper 1/2. Generally, the villi were stained all over except at the bases. One dome-like area was observed in the upper portion of the jejunum.

The villi in the ileal segments varied from tall, cylindrical with rounded tips, to short stunted with pointed tips. The latter were only palely stained. The short villi became fused as groups of 2 villi. The fusion of villi was a predominant feature of the distal segment where villi appeared in larger groups of 3-8 villi. In this segment, some individual short villi were plump with pointed tips. Few branching villi were observed in the proximal segment of the ileum and some irregular villi were scattered in this section.

GC 38

The villi in the duodenum were uniform, tall to moderate in length and flat, cylindrical or leaf-like in appearance. The tips were rounded or slightly elongated. The villi were stained uniformly.

The jejunum had regular villi, tall, flat or cylindrical with rounded tips. However, a few short villi and elongated tips were observed. Some branching villi were observed along the segments. Several dome structures were located in different areas of the jejunum.

The ileal villi were also regular, tall with flat or cylindrical appearance and rounded tips. Some short villi were observed. There were 2 areas of dome structures in the proximal segment of the ileum 120.

GC 41

The villi of the duodenum were moderate to tall. The villi were flat and uniform with rounded tips. The stain was taken uniformly but some tips were darkly stained. Several dome structures were distributed all over the segment.

In the jejunum, villi were tall and tended to be shorter in the last segment of the jejunum 700. Generally, the villi were flat or cylindrical in shape with rounded tips. Few villi were pointed or slightly enlarged in the jejunum 700. The stain was taken uniformly although some villi were stained darkly in the upper 1/2. Several dome structures were observed in all the segments of the jejunum but 100. Some branching villi were detected in the jejunum 100 and 320.

The ileal villi were moderate in length and flat or cylindrical in shape. The tips of the villi were rounded and some were pointed. The stain was taken up uniformly by the villi. One area of dome structures was observed in the proximal segment.

Examination of the whole intestinal mucosal surface by the methylene blue stain proved most useful in the demonstration that the sections taken from determined sites in fact represented the lesions occurring in these regions. The sections, of course, only represent regions 6 microns thick.

Wet Tissue Measurement

Figures 13a and 13b show results of the wet tissue measurements of villous height and cryptal depth of the infected calves compared to the average of the 2 control calves. The data revealed that there was no obvious reduction in villous lengths in GC 16 compared to the average of 2 control calves. When the other 3 infected calves were compared to the 2 controls, there was marked shortening of villi in both ileum sites of all 3 calves and in the lower jejunum of GC 18 and GC 20. The depth of crypts in some sites of the upper small intestine of GC 14, GC 18 and GC 20 exceeded the crypt depths of the controls. The crypts in ileum 50 of GC 14 and GC 18 were deeper than in the controls.

Lesions induced by Breda virus I

Many of the observations recorded before applied to both control and infected calves must be considered normal. The upper small intestine, including duodenum through jejunum 450, except calf GC 18, appeared

Figure 13a. Measurements of villous length and crypt depth in 3 experimentally inoculated gnotobiotic calves [for comparison to average of measurements from 2 controls and GC 16, see Figure 13b]

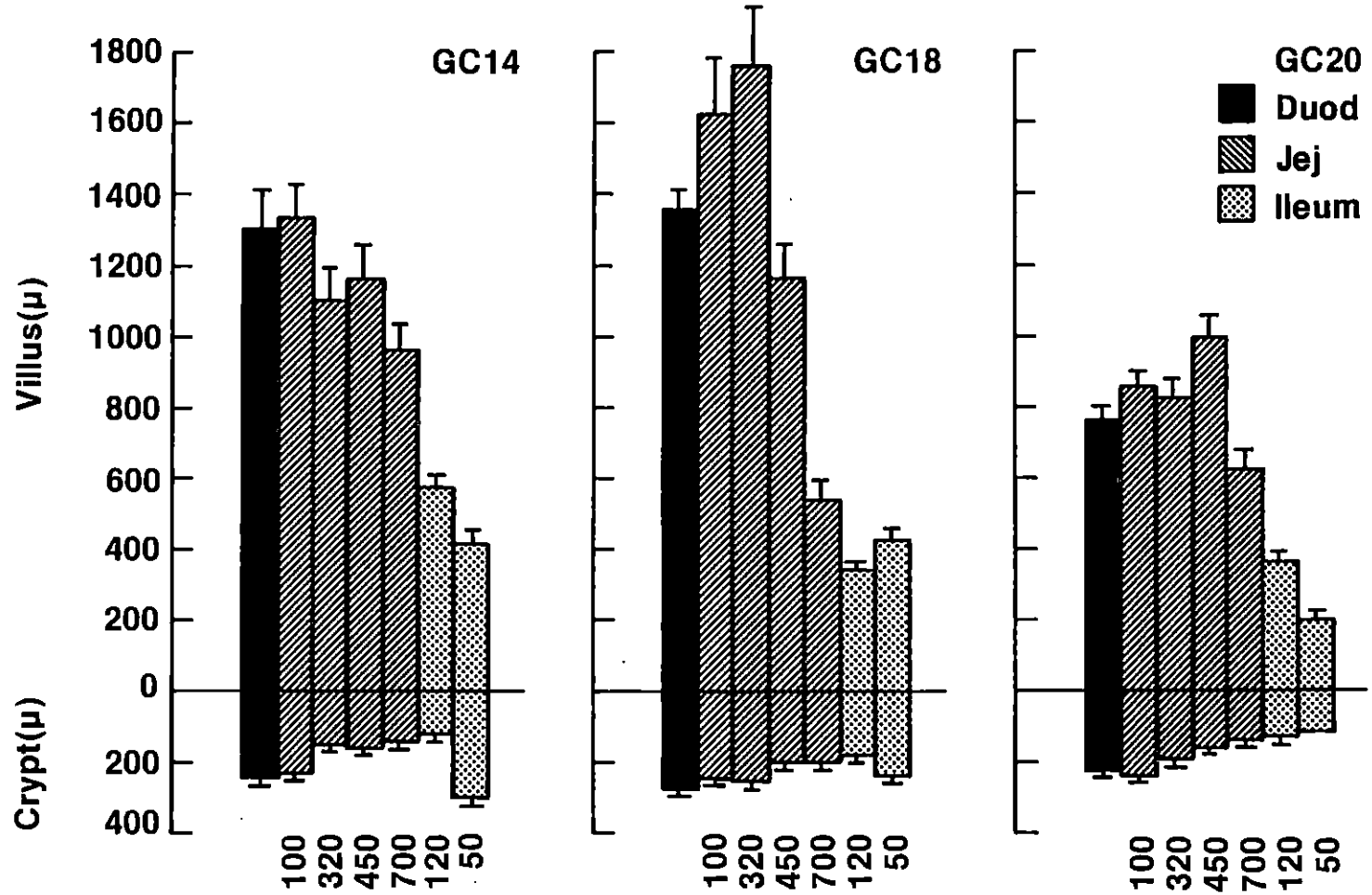
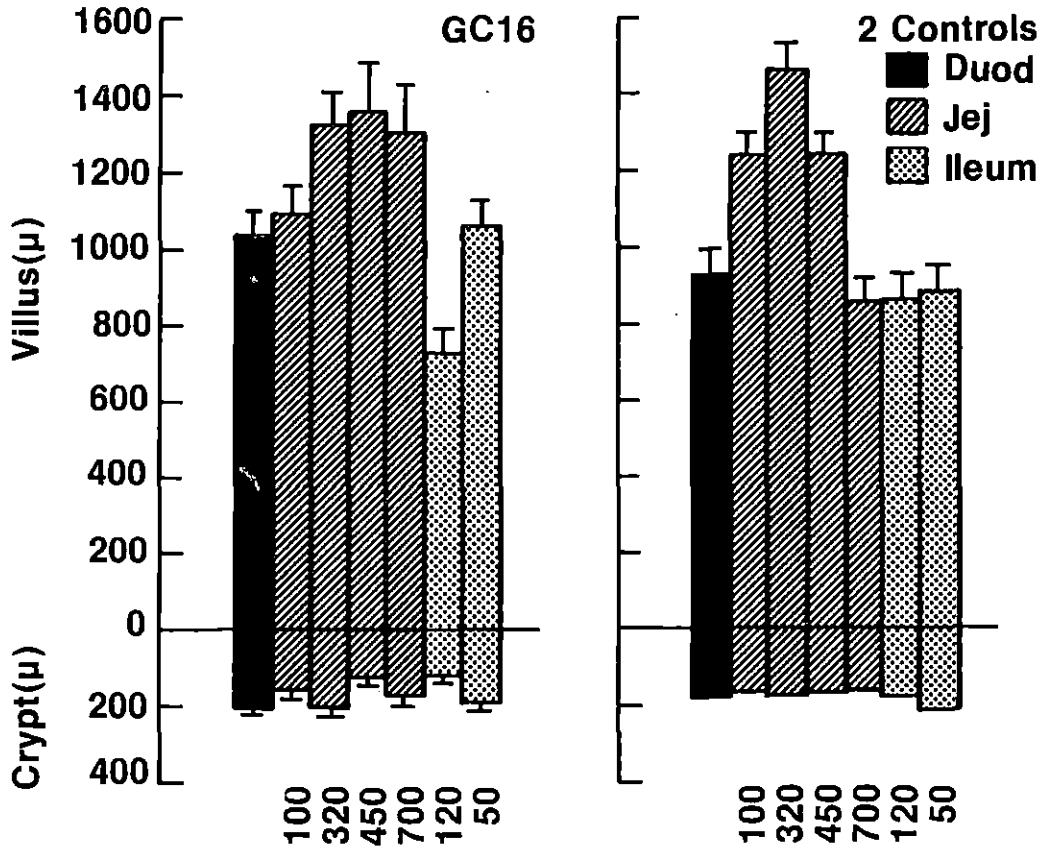

GC14
GC18
GC20
Duod
Jej
Ileum
Villus(μ)
Crypt(μ)
1800
1600
1400
1200
1000
800
600
400
200
0
200
400
100
320
450
700
120
50

Figure 13b. Measurements of villous length and crypt depth of calf GC 16 compared to 2 control calves [for comparison to GC 14, GC 18 and GC 20, see Figure 13a]


GC16
2 Controls
Duod
Jej
Ileum
Villus(μ)
Crypt(μ)
1600
1400
1200
1000
800
600
400
200
0
200
400
100
320
450
700
120
50
100
320
450
700
120
50

normal. Dilation of lacteals and lymphatic vessels, congestion, a few crypt abscesses and infiltration of eosinophils were observed in all calves including the controls in all sites of the intestine.

The consistent changes induced by Breda virus I were manifested as severe villous destruction, villous atrophy, replacement of epithelial cells by immature cells, crypt hyperplasia, increased numbers of crypt abscesses and necrosis of cells in villous bases and crypts with varying numbers of macrophages containing cellular debris in lamina propria.

The Electron Microscopic Study

The electron microscopic study revealed the presence of acute viral infection of the lower small and the large intestine. The lesions induced by Breda virus I correlated well with viral detection either by electron microscopy or indirect immunofluorescence (Table 2). The virions in infected cells were elongated with rounded ends, and measured 35 nm x 80 nm (Figure 16b). Short projections were associated with the outer membrane and they surrounded an internal double membrane core of 25 nm in diameter.

The lesions were mostly observed in the lower jejunum, ileum and large intestine of calves GC 14, GC 18 and GC 20 killed at 48 hrs, 95 hrs and 93.5 hrs P.I. However, GC 16, which was killed 36 hrs P.I., had no detectable viruses in the feces. This calf had slight immunofluorescence of viral antigen in a few cells damaged by the virus only in jejunum 450. The lesions in the other 3 calves were observed in absorptive epithelial

cells, in crypt epithelial cells and in macrophages of lamina propria with the presence of viral particles (Figure 14).

The affected epithelial cells had different degrees of cell degeneration. The microvilli were irregular, short and were sometimes stunted and fused (Figure 14). The viral particles were found between these disrupted microvilli (Figure 15). The affected epithelial cells underwent lesions from acute swelling and hydropic degeneration to dilation and fragmentation of the endoplasmic reticulum. Golgi complex reduplication and mitochondrial irregularity were also observed. Membrane bound autophagolysosomes were numerous in infected cells. These contained cellular debris and viral particles. The viral particles were also found in membrane bound vacuoles (Figures 14, 15, and 16b) or free in the cytoplasm. Degenerated epithelial cells were sloughed into the lumen in some areas (Figures 16a and 16b).

Villous fusion was found in most severely affected calves as it was more pronounced in the later stages. The fused villi appeared as long broad ridges with clustering of epithelial cells at the fusion sites. The epithelial absorptive cells were sometimes separated by broad irregular spaces which were interpreted to be intercellular edema. Although the cytopathic changes involved most cellular components, the intercellular junctions were intact in the early phase of infection and the desmosomes were not altered. In most severely infected areas, affected cells were vacuolated and cellular junctions were less dense.

Lamina propria macrophages were enlarged and contained cellular debris, myeloid bodies and viral particles (Figure 14). Tubular structures

Figure 14. Ileum 50 of GC 14 (48 hrs P.I.): 1) infected epithelial cell with groups of virions in membrane bound vacuoles [arrows] and disrupted irregular short fused microvilli [arrowheads], 2) macrophage beneath the basement membrane containing autophagolysosomes (6000x)

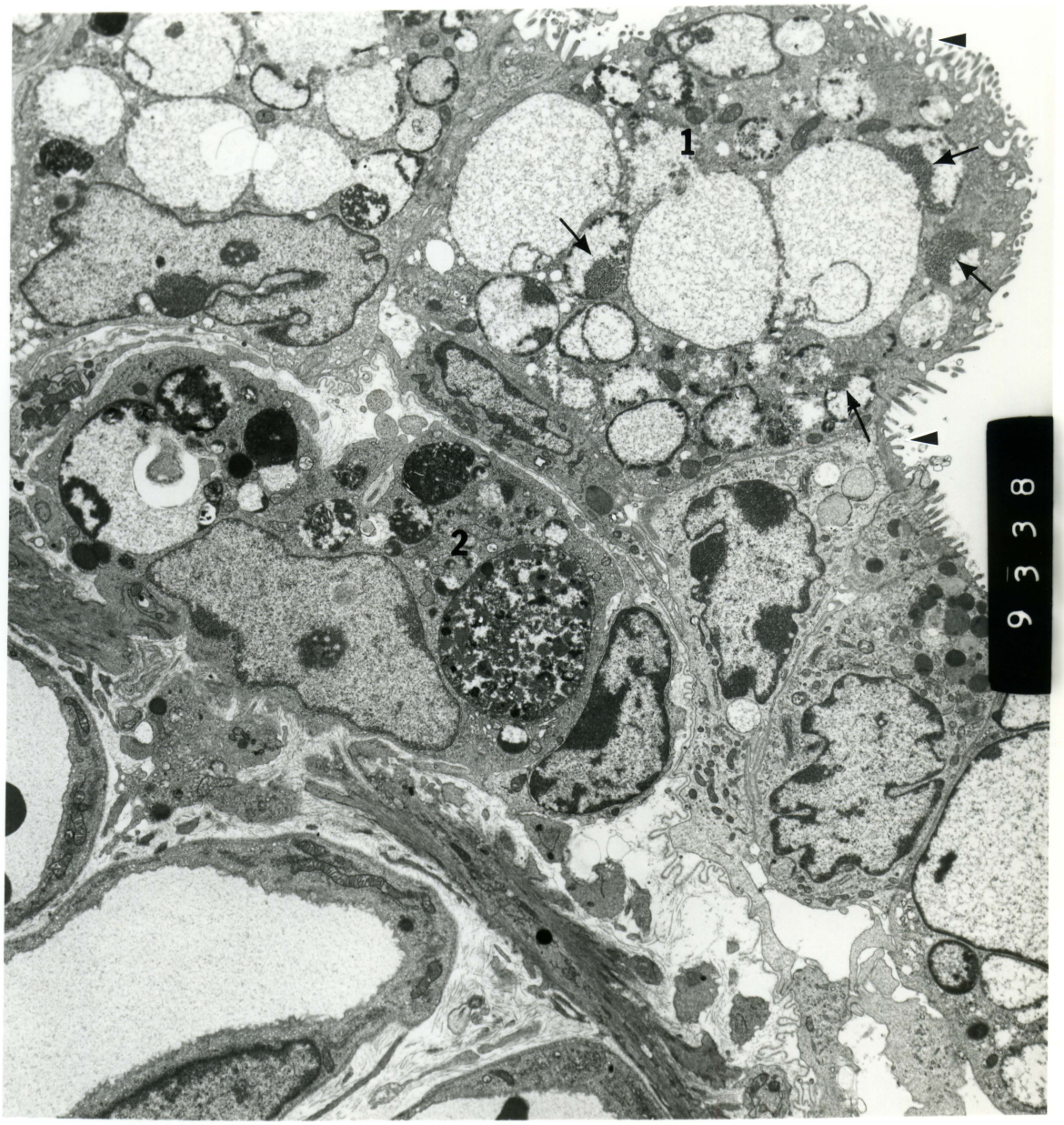

1
2
9338

Figure 15. Descending colon of GC 18 (95 hrs P.I.). Viral particles in between short, stunted microvilli of infected epithelial cell [arrow]. Intracytoplasmic membrane bound vacuoles containing single or a group of virions [arrowheads] (40000x)

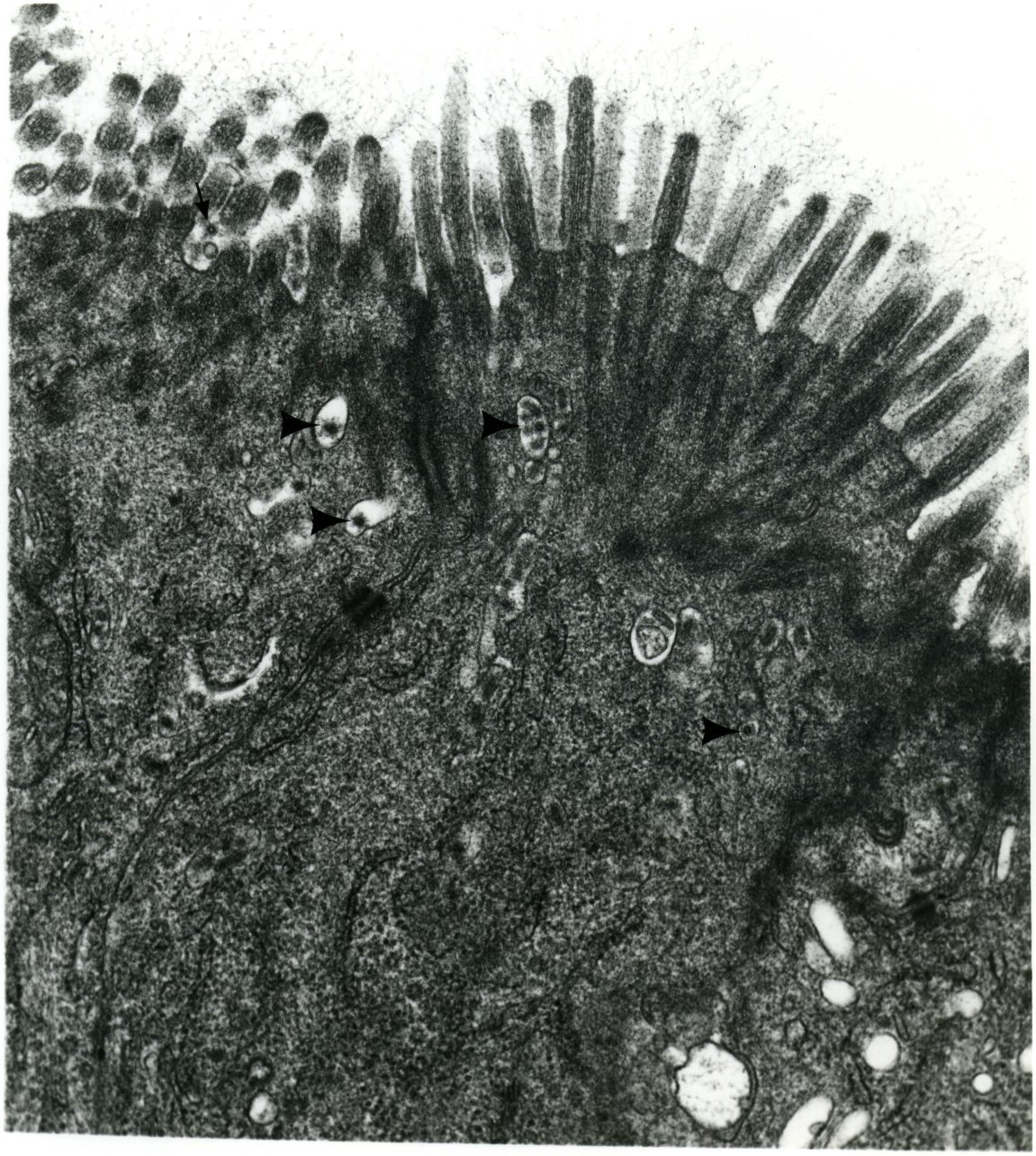
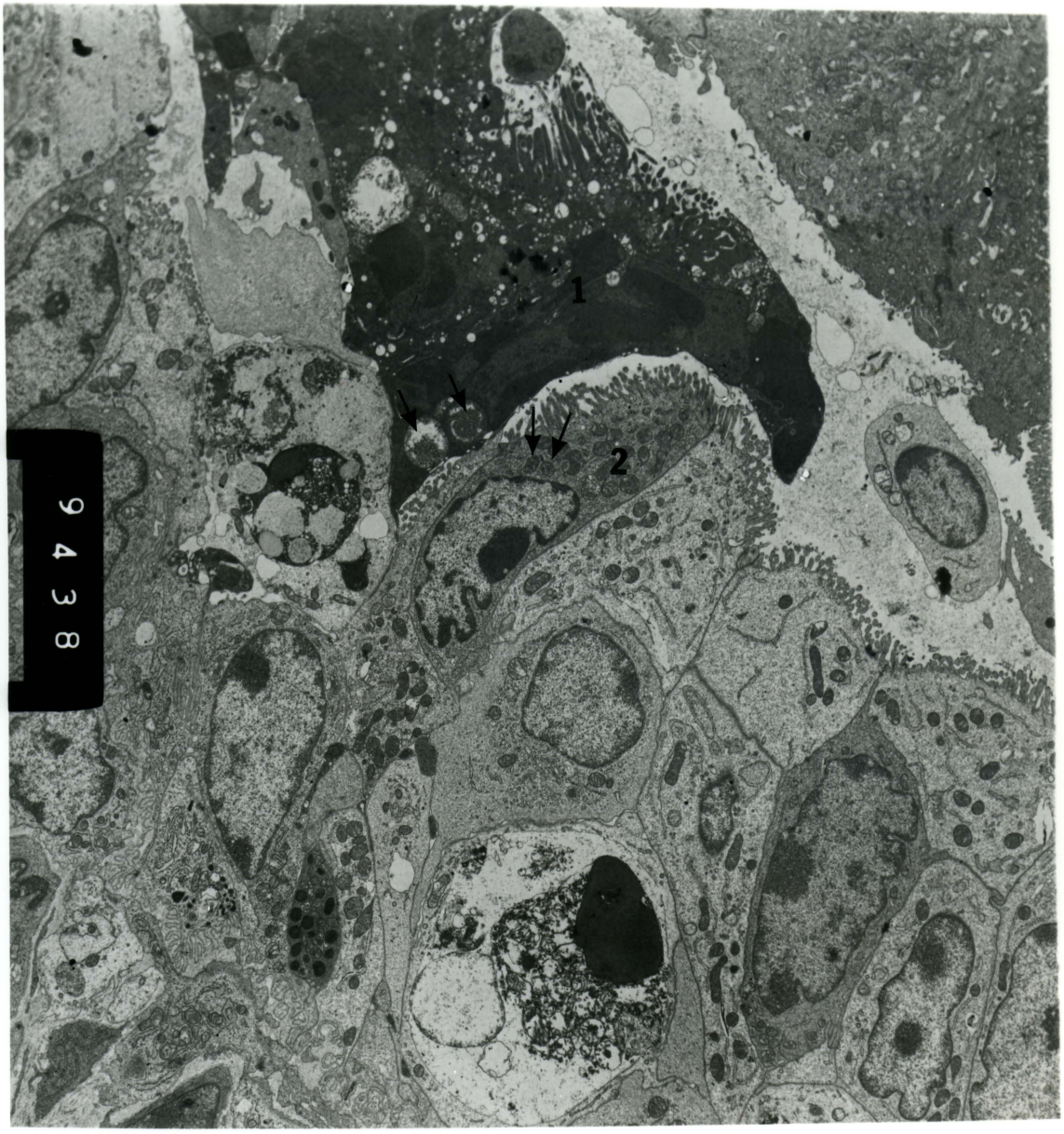


Figure 16a. Ileum 50 of GC 14 (48 hrs P.I.): 1) electron dense necrotic epithelial cell sloughing into the lumen containing 2 less dense vacuoles [arrows] with viral particles, 2) epithelial cell still connected to other cells on the surface containing membrane bound virus particles [arrows]. [Higher magnification in Figure 16b] (6300x)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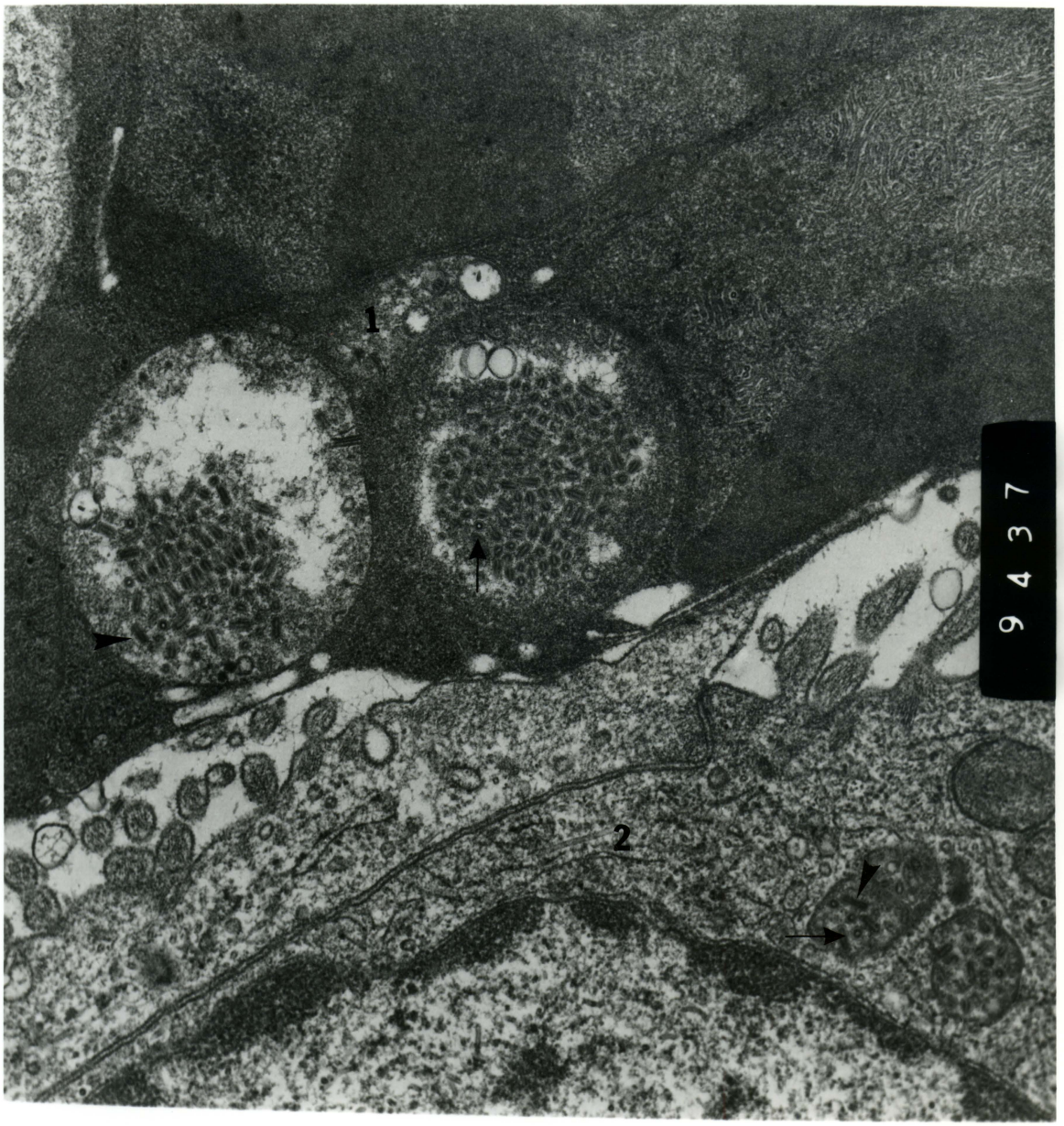
9438

Figure 16b. Ileum 50 of GC 14 (48 hrs P.I.): membrane bound vacuoles containing viral particles in 1) necrotic sloughing epithelial cell, 2) infected epithelial cell with irregular disrupted microvilli. Round viral particles [arrows]. Elongated viral particles with rounded ends [arrowheads] (40000x)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2
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were found in infected epithelial cells and macrophages. They were prominent in the autophagosomes and it has been suggested that these tubules were probably associated with viral replication (Pohlenz et al., 1984).

Histology of the Parenchymal Tissues

The thymus in all infected and control calves was infiltrated with low numbers of eosinophils. These were mainly observed in the medulary and interlobular areas with a few cells present in the cortical areas. The liver of GC 14 and GC 20 had mild infiltration of neutrophils in the hepatic parenchyma. Neutrophils were packed in some liver blood vessels of GC 14. The GC 16 had moderate diffuse bile retention in the hepatocytes. There was no change observed in the forestomachs of any of the animals except the presence of multifocal serosal hemorrhage of the abomasum in GC 14 and GC 16. In addition, serosal fibrin deposition on abomasum of GC 14 was found. These lesions were not associated with any inflammatory reactions and were interpreted to be due to the necropsy procedure. The kidneys of GC 14 and GC 38 had mild thickening of Bowman's capsules. Acute mild focal aspiration pneumonia with the presence of a few neutrophils in alveolar and bronchiolar lumens was observed in GC 16 and GC 20. GC 41 had multifocal alveolar thickening with mild infiltration of eosinophils. There was mild diffuse medullary infiltration of eosinophils in the mesenteric lymph node of GC 20 and GC 41 which also had severe cortical lymphoid depletion. No histologic changes were observed in the other organs taken from the calves.

DISCUSSION

The results confirm previous publications that the unclassified Breda virus I is a pathogen in gnotobiotic calves. The predominant lesions were restricted to the lower jejunum and the ileum and were marked in the large intestine. The changes were characterized by necrosis of villous absorptive cells, villous atrophy, villous fusion and crypt hyperplasia in later stages of infection. Crypt epithelial necrosis was prominent in the lower small intestine and large intestine.

The inoculated calves showed variation in time of onset of diarrhea and virus excretion following inoculation, and thus, the animals represented a sequence of events. Calf GC 16 which was necropsied 36 hrs P.I. did not develop diarrhea or any clinical signs and had no detectable virus in the feces (Table 1). This may suggest that the calf was within the incubation time and developed only the early phase of infection as demonstrated by immunofluorescence to Breda virus antigen in one site of the small intestine (jejunum 450). Viral particles were also observed at the same site by electron microscopy. GC 14 commenced diarrhea at 46 hrs P.I. and it was killed 2 hrs later when it started shedding the virus in the feces. The clinical signs manifested as depression and anorexia accompanied by muscular shivering indicating an acute stage of viral infection. The other 2 calves, GC 18 and GC 20, developed diarrhea after 48 and 60 hrs P.I. and were killed 95 and 93.5 hrs P.I., respectively (Table 1). This indicated that the incubation period in these 3 calves ranged from 46-60 hrs when pathological changes were also observed in the

intestinal tract. The difference observed between individual animals in the time related to onset of diarrhea and lesions suggests a significant amount of animal variation.

Histologically, the upper parts of the small intestine including the duodenum through jejunum 320 had no changes. However, in calf GC 14 at jejunum 320 and in calf GC 16 at jejunum 450, fluorescence for Breda virus I was positive although no histological lesions were detected. The presence of round, less intensely stained enterocytes containing round nuclei on tips of some villi were not observed in our control calves. However, they were reported in uninfected gnotobiotic calves (Mebus et al., 1975a). Enlargement of some tips of villi in the upper small intestine in most of the animals including the controls may have resulted from dilation of capillaries and lacteals. This may be an artifact possibly induced by the autopsy procedure.

The severity of lesions in the lower jejunum and ileum varied between the infected animals from severe surface destruction with epithelial necrosis to villous atrophy, extensive villous fusion, and replacement of columnar epithelial cells with cuboidal to squamous epithelial cells. Most likely, the contraction of the lamina propria that reduced villous length and expansion of immature cells in an attempt to cover the denuded areas are associated with villous atrophy as described by Moon (1983) to occur in intestinal virus infection. Doughri and Storz (1977) considered villous fusion to result from the reduced motility of the shortened villi and their tendency to adhere to each other. It is possible, however, that

the presence of virus in the plasmalemma of infected cells at the site of the fusion and immature epithelial cells replacing the necrotic cells participate in the fusion process. Similar fusion and villous atrophy have been described in calves infected with coronavirus (Mebus et al., 1975b; Doughri and Storz, 1977; Mebus, 1978), with Newbury agent (Hall et al., 1982), and with Cryptosporidium sp. (Pearson and Logan, 1978; Pohlenz et al., 1983). Stunted and fused villi were predominant lesions in severely affected colostrum-deprived calves with rotavirus (Logan et al., 1979). Villous fusion was demonstrated well by examination of methylene stained whole intestine and villous atrophy was best recorded by villous measurements on wet tissues. Villous atrophy and villous fusion were not observed in GC 16. This animal was killed in the early stage of infection and morphological changes in villous architecture had not yet occurred.

In the lower jejunum and ileum, the presence of necrotic cells in the bases of villi and in crypts as well as large debris containing macrophages have not been described in association with viruses causing acute neonatal calf diarrhea in gnotobiotic animals. Since Breda virus I particles or antigen were detected by electron microscopy and immunofluorescence it is concluded that these changes resulted from Breda virus I infection. These observations suggest that there may be primary virus infection in crypt epithelial cells and cells of the lower part of the villus. Pyknotic cells in the lamina propria probably similar to those macrophages in Breda virus infection were described in coronavirus-infected

calves (Mebus et al., 1973; Mebus et al., 1975b), rotavirus-infected calves (Woode et al., 1974) and in infection with calicivirus-like agent (Hall et al., 1982).

Breda virus was detected either by electron microscopy of immunofluorescence in all sites of the large intestine of infected calves except in calf GC 16 killed 36 hrs P.I. Histologically, there was multifocal epithelial necrosis, sloughing of epithelial cells or replacement with cuboidal cells on the surface. These lesions were more severe in the later stages of infection, 95 and 93.5 hrs P.I. Similar changes were described in the colonic epithelium of coronavirus-infected calves (Mebus et al., 1973; Mebus et al., 1975b; Doughri and Storz, 1977; Mebus, 1978). No colonic lesions have been reported in association with Newbury agent (Woode and Bridger, 1978), astrovirus (Woode et al., in press) or E. coli (Runnels et al., 1980). No evidence of large intestinal infection has been shown in calf rotavirus infection. However, a few immunofluorescent cells were found in the large intestine of rotavirus-infected lambs (Snodgrass et al., 1977).

The lesions in deep mucosa of the large intestine of GC 18 and GC 20 consisted of multifocal crypt cell necrosis which was occasionally observed at crypt orifices. The crypt cell necrosis was more severe in the colon than in the cecum. In addition, there were debris-laden macrophages in the lamina propria of the colon. These findings have not been reported with other viral infections of gnotobiotic calves with the exception of coronavirus infection (Mebus et al., 1973; Mebus et al., 1975b) where there was

loss of goblet cells and replacement of crypt epithelium with cuboidal cells.

Eosinophilis were present in lamina propria of the control calves and in all 4 experimentally infected calves in all sections taken. The number of cells was less in control than in infected animals and it seemed that there were fewer eosinophils in the large intestine than the small intestine. It is not certain whether the presence of these cells in the 4 inoculated calves is due to Breda virus I infection. The presence of eosinophils was considered to be a normal finding (Mebus et al., 1971). Eosinophils were observed in the lamina propria of control calves and rotavirus inoculated calves. These cells were also reported in control and chlamydial-infected calves (Eugster et al., 1970) and in the calves of an experiment with E. coli infection (Pearson et al., 1978a).

There were increased numbers of mitotic figures in all sites of the intestinal tract of GC 18 and GC 20, except in the cecum of GC 18, compared to the control calf GC 38 (Table 3). The other control calf, GC 41, had high numbers of mitotic figures. The presence of increased mitotic figures in this animal is unexplained. There were no infectious agents detected in this animal and no mucosal destruction was found. It is concluded that the increase of mitotic figures in GC 18 and GC 20 is a response to the loss of epithelial cells and this is interpreted to be a regenerative response which occurred especially in the lower jejunum, ileum

and large intestine. There was no consistent increase in mitotic figures in the GC 14 and GC 16 compared to the control calves. GC 14 and GC 16 were necropsied in the early phase of infection (48 and 36 hrs P.I.) when no increase in mitotic figures is expected. An increase in crypt depth was noticed in some sites of the upper small intestine of GC 14, GC 18 and GC 20 (Figure 13a) and in both sites of the ileum of GC 14 and GC 18. This increase may be explained as a response to mucosal damage. Currently, there are no methods available to measure stimulating effects on the renewal of intestinal mucosa. Enteroglucagon was discussed by Bloom (1979) as a growth hormone of the gut in the human. There was no possibility to evaluate this hormone in this experiment.

Varying numbers of crypt abscesses were observed in the small intestine of all calves, infected and control calves. Crypt abscesses were characterized by dilation of crypts which were lined by low columnar to cuboidal or flat epithelial cells with lumenal cellular debris, granular or amorphous eosinophilic material and numbers of inflammatory cells. These abscesses were suggested to result from occlusion of crypt orifices by exudate and denuded epithelial cells (Doughri et al., 1974). These authors also discussed that degeneration of crypt cells may result from an increase of pressure within crypt lumens generated by the occlusion. These cryptal changes were observed in the small and the large intestine of conventionally reared calves infected with coronavirus (Doughri and Storz, 1977). Crypt abscesses were described in the ileum of infected calves with cryptosporidium (Meuten et al., 1974; Morin et al., 1976).

Viral detection in the lower jejunum, ileum and large intestine were almost consistent by using electron microscopy and immunofluorescence in animals GC 18, GC 20 and GC 14. In GC 14, there were 3 sites, jejunum 320, cecum and spiral colon, where only virus antigens were detected by immunofluorescence. In GC 16, killed 36 hrs P.I., the virus was present only at site 450 of the small intestine. This observation indicates that virus replication occurs predominantly in the lower small intestine and the large intestine.

The Breda virus I characteristics appeared to be different from other known bovine enteric viruses. At superficial examination, Breda virus I may be confused with coronavirus (Pohlenz et al., 1984). However, Breda virus I has short 7-9 nm long projections compared to coronavirus 17-24 nm long projections (Bridger et al., 1978). In addition, it has an internal double membrane core which measures 25 nm in diameter. The virions in infected cells measure 35 x 80 nm. Diameters of the virions are different from coronaviruses (70-80 nm) (Mebus et al., 1973; Doughri et al., 1976), rotaviruses (50-69 nm) (Woode et al., 1976), adenoviruses (70-90 nm) (Gillespie and Timoney, 1981), Newbury agent (33 nm) and astrovirus (30 nm) (Woode and Bridger, 1978), and parvoviruses (18-25 nm) (Kahrs, 1981).

Diarrhea in infected calves was most likely due to maldigestion and malabsorption. The epithelial damage in the early phase of infection or replacement of absorptive epithelial cells by immature cells in the later phase of infection decreases the absorption and digestion of the ingesta

in the small intestine. The ingesta are passed into the large intestine where incompletely digested materials increase the osmolality. The presence of these materials tends to hold water in the lumen and draw water from the circulation which enhances diarrhea (Moon, 1978).

COMPARISON BETWEEN BREDA VIRUS I INDUCED LESIONS AND OTHER COMMON ENTERIC NEONATAL DISEASES IN GNOTOBIOTIC CALVES

Morphologic changes induced by Breda virus I were prominent in the lower jejunum, ileum and large intestine. These lesions included necrosis of villous surface, villous atrophy, villous fusion and crypt hyperplasia. Necrosis of epithelial cells in the bases of villi and crypt cell necrosis with the presence of macrophages containing cellular debris were found throughout the infected sites.

1. Lesions induced by calf coronavirus are predominant and consistently found in the middle and lower small intestine. The proximal small intestine and the large intestine are affected to a lesser degree. Necrotic crypt cells have not been described in the small intestine but do occur in the large intestine.

2. Lesions associated with rotavirus infection are described in the small intestine only. The lower part of the small intestine is affected later in the course of infection than the upper part of the small intestine. Predominant lesions occur in the jejunum. Rotavirus infects mature absorptive epithelial cells only and no crypt cells.

3. Calici-like agent (Newbury agent) causes lesions in the jejunum and anterior ileum only and no lesions in the large intestine.

4. Bovine astrovirus was shown to possess an affinity for dome epithelial cells inducing degenerative changes.

5. E. coli microorganisms colonize the villi of the middle and lower small intestine. No changes have been found in the crypts of infected sites or in the large intestine. Most strains of enterotoxigenic E. coli do not produce any morphologic lesions.

6. Cryptosporidium sp. is found throughout the small and large intestine. Villous atrophy, villous fusion and crypt hyper-

plasia are described in the small intestine. The presence of the organisms along with lesions is characteristic. The organisms are present in the large intestine accompanied by mild inflammatory response.

These comparisons demonstrate that lesions induced by Breda virus I are different and distinguishable by morphologic investigation.

SUMMARY

Four gnotobiotic calves were inoculated intranasally with Breda virus I preparation at 1-2 hrs of life. The unclassified Breda virus was described by Woode et al. (1982). The inoculated calves GC 14, GC 16, GC 18 and GC 20 were necropsied at 48 hrs, 36 hrs, 95 hrs and 93.5 hrs P.I., respectively. Two gnotobiotic calves were kept as controls and were necropsied on day 5 of age. Calf GC 16, necropsied at 36 hrs, did not develop diarrhea and Breda virus was not detected in the feces. The other 3 infected calves, GC 14, GC 18 and GC 20, developed diarrhea at 46 hrs, 48 hrs and 60 hrs P.I. and Breda virus was present in their fecal samples. GC 16 had no detectable lesions other than a few infected cells in one site, jejunum 450, as shown by immunofluorescence and electron microscopy. The lesions in the other 3 infected calves were found in the lower jejunum, ileum and large intestine. These lesions were characterized by villous destruction, villous atrophy, and villous fusion. Villous fusion was determined by examination of histologic sections and methylene blue stained preparations. Villous length and crypt depth measurements on wet tissues revealed marked reduction of villous lengths in both ileum sites of GC 14, GC 18 and GC 20, and in the lower jejunum of GC 18 and GC 20. There was also necrosis of epithelial cells in the bases of villi and in crypt epithelial cells. High numbers of macrophages containing cellular debris were present in most affected sites. Crypt hyperplasia was more pronounced in GC 18 and GC 20. Breda virus I particles or viral antigen were detected by either electron microscopy or immunofluorescence

in villous epithelial cells and crypt cells of almost all sites of the lower jejunum, ileum and large intestine of GC 14, GC 18, and GC 20. Electron microscopic examination showed that most infected cells underwent hydropic degeneration with dilation and fragmentation of endoplasmic reticulum. Viral particles were mostly found in membrane-bound vacuoles.

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