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Enteric Lesions and Diarrhea in Gnotobiotic Calves Monoinfected with *Cryptosporidium* Species

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The pathogenicity of *Cryptosporidium* species was studied by inoculation of two gnotobiotic calves with cryptosporidial oocysts that had been decontaminated by treatment with peracetic acid. Two control calves were inoculated with similar material from which the oocysts had been removed by filtration. Oocyst-inoculated animals shed *Cryptosporidium* in their feces and developed depression, weakness, anorexia, and diarrhea. At necropsy five days after inoculation, endogeneous stages of *Cryptosporidium* were found in association with epithelial cells throughout the small and large intestines of these animals. The parasites were most numerous in the lower small intestine. Atrophic villi, disordered and degenerate villous epithelium, and hyperplastic crypt epithelium were associated with infection in the small intestine. Control animals remained normal. Extraneous agents were not detected in any of the calves. The results indicate that *Cryptosporidium* can destroy intestinal epithelial cells and cause diarrhea in monoinfected gnotobiotic calves.

Infections with the coccidian parasite *Cryptosporidium* occur worldwide in the alimentary and respiratory tracts of numerous species of vertebrates. The infection is highly prevalent among cattle; it affects up to 100% of calves during the first four weeks after birth [1–5]. Subclinical infection is common; however, infection is also frequently associated with diarrhea in calves and people [5–10]. Cryptosporidiosis has recently been recognized as a zoonosis [10–12]. The infection is self-limiting in calves and people with normal immune systems. However, it can become persistent in immunologically compromised individuals, such as patients with the acquired immunodeficiency syndrome [12–14]. Clinically affected calves have atrophy of villi and hyperplasia of crypt epithelium (apparently as a result of the destruction of villous epithelium); those areas of the small intestine that are heavily infected with the parasite become inflamed [7, 9, 10]. Some calves also have epithelial damage and inflammation associated with numerous parasites in the large intestine [7]. Similar lesions occur in people with cryptosporidiosis [15–17].

The diarrhea and intestinal lesions associated with cryptosporidiosis in calves and people are presumed to be caused (at least in some cases) by the parasite. However, in calves *Cryptosporidium* frequently coexists with enteropathogenic viruses, bacteria [5], or other protozoa known to be independently capable of causing these signs and lesions. Although the development of signs and lesions during intraspecies and interspecies transmission experiments with *Cryptosporidium*, in the absence of other recognized enteropathogens, suggests that the parasite causes these changes [7, 10], the possibility of cotransmission of some unrecognized pathogens in such experiments cannot be excluded. Moreover, experimental infections in conventional animals have not served to clarify whether or not the intestinal flora is required for colonization and lesion production by

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the parasite. On the other hand, if confirmed, the reported occurrence of diarrhea and intestinal lesions in gnotobiotic pigs infected with an inoculum treated in a manner that destroys infectious agents other than *Cryptosporidium* [18] provides strong evidence that the parasite can act as a primary enteropathogen in the absence of other enteric flora.

The data reported here tend to confirm the report that *Cryptosporidium* can cause diarrhea and intestinal lesions in monoinfected gnotobiotic animals. These studies extend the data to another host species and involve a different isolate of *Cryptosporidium*, a different treatment for the destruction of extraneous agents, a comprehensive search for such agents, and the use of control animals exposed to putative, unrecognized viruses that might have survived the treatment intended to destroy these agents.

Materials and Methods

Calves. Four gnotobiotic calves were delivered by cesarean section at 270–274 days of gestation and were maintained as described by Matthews et al. [19]. The calves were each fed 2 liters of reconstituted condensed milk twice daily. The isolators were kept at 18 C–24 C.

Inocula. Feces from two calves experimentally infected with *Cryptosporidium* of calf origin were suspended in two volumes of 2.5% potassium dichromate solution. This suspension was passed through a sieve (mesh size, 63 μm) and stored for 10–15 weeks at 4 C. The suspension contained 7.5 × 10^6 cryptosporidial oocysts/ml, as determined by the counting technique reported previously [4].

In preliminary tests cryptosporidial oocysts (in potassium dichromate suspensions of calf feces) that were treated with 3.2% peracetic acid (vol/vol) and held at 22 C for 20 min retained infectivity for mice. Oocysts were removed from peracetic acid by centrifugation (500 *g* for 10 min) and three aseptic washes (by centrifugation) of the sediment in PBS. Such peracetic acid–treated oocyst preparations yielded no bacterial or fungal growth when cultured aerobically at 37 C for one week in trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md). In view of these results and the facts that peracetic acid is routinely used as a germicide in gnotobiotic procedures and is effective against a broad spectrum of microbes, this treatment was used for the destruction of putative microbes other than *Cryptosporidium* in the inocula given to gnotobiotic calves.

Each of two calves (no. 1 and no. 2) received the sediment (resuspended in 10 ml of PBS) resulting from peracetic acid treatment of 13.5 ml of potassium dichromate–suspended feces (10^8 oocysts per calf). Inocula for two control calves (no. 3 and no. 4) were prepared from the same potassium dichromate suspension of feces (27 ml of suspension per calf). The control inocula were passed twice through filters (pore size, 0.8 μm) for the removal of cryptosporidial oocysts before treatment with peracetic acid. The inocula were mixed with the milk fed to the calves at 22 hr of age.

Aliquots of the oocyst-containing and control inocula were given to baby mice as a test for the presence of infective *Cryptosporidium* and were cultured for aerobic bacteria at 37 C in TSB. None of the inocula produced detectable growth in TSB, and only mice given the oocyst-containing inocula became infected with *Cryptosporidium*.

Observations. All four calves were observed at 12-hr intervals for five days after inoculation. Rectal temperature, appetite, strength, attitude, and character of feces were recorded at each interval. Fecal samples were collected at each interval and examined microscopically for oocysts by the carbol fuchsin technique [23]. The percentage of fecal dry weight was determined by drying of each sample to a constant weight at 100 C.

In addition, a daily fecal sample was cultured for aerobic bacteria at 37 C for 24 hr in TSB. A fecal sample obtained from each calf on day 5 after inoculation was also cultured anaerobically at 37 C for 48 hr on sheep blood agar and in chopped meat broth. Direct smears were made from the final fecal sample obtained from each calf. These smears were stained with gram stain and examined microscopically for bacteria. Daily fecal samples were examined for bovine viruses (coronavirus, rotavirus, astrovirus, and Breda virus by methods reported previously [20–22] as well as by direct electron microscopy of negatively stained preparations. No viruses or bacteria were detected in any sample.

Blood samples were taken from each calf immediately before inoculation and 72, 96, and 120 hr afterwards. The following values were determined by routine procedures: packed cell volume; erythrocyte count; total and differential leukocyte counts; concentrations of hemoglobin, plasma

Table 1. Distribution of *Cryptosporidium* in histological sections from the intestines of gnotobiotic calves.

Inoculum, calf no.	Cryptosporidial distribution in indicated intestinal section*									
		Jejunum				Ileum			Colon	
	Duodenum	100	320	450	700	120	50	Cecum	Spiral	Descending
10^8 oocysts										
1	+	+ +	+ +	+ +	+ + +	+ + +	+ + +	+	+	–
2	+	+	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	–
Control										
3	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–

* Sections 6 μm thick were prepared 120 hr after calves no. 1 and no. 2 were inoculated with cryptosporidial oocysts. The numbers under the headings "jejunum" and "ileum" represent centimeters distal to the duodenal site (jejunum) or proximal to the ileocecal valve (ilcum). Key: – = no demonstrable parasites; + = <20 parasites/mm of epithelial surface; + + = 20–80 parasites/mm; and + + + = >80 parasites/mm.

protein, albumin, fibrinogen, calcium, inorganic phosphorus, urea nitrogen, Na^+, K^+, and Cl^-; and aspartate aminotransferase level.

Calves were anesthetized with barbiturate 120 hr after inoculation. Two ligated segments of intestine were created at each of the following sites: duodenum (15 cm distal to the pylorus), jejunum (100, 320, 450, and 700 cm distal to the duodenal site), ileum (50 and 120 cm proximal to the ileocecal valve), cecum, apex of spiral colon, and descending colon. The ligated segments were instilled intraluminally with fixative (10% formalin for one segment and 3% glutaraldehyde for the other), excised surgically, and placed in fixative. Calves were killed after these segments were collected. Portions of each formalin-fixed segment were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin for the preparation of histological sections. Portions of formalin-fixed small intestine were also stained by immersion in 2% methylene blue. "Whole-mount" preparations of mucosa (one or two villi thick and four to 10 villi long) were dissected from the methylene blue–stained segments with use of a dissecting microscope, a razor blade, and straight pins; the dissection was done underwater by a modification of the procedure of MacDonald and Ferguson [24]. These preparations were mounted on glass slides in water, with the long axis of villi parallel to the surface of the slides. The lengths of the villi and depths of the crypts in the whole mounts were measured with a dissecting microscope equipped with an ocular micrometer. Glutaraldehyde-fixed sections of the small intestine were prepared for and examined by scanning and transmission electron microscopy according to routine procedures.

The number of parasites per unit (length) of epithelium in histological sections was determined microscopically with a semiautomatic computerized digitizing tablet (Bioquant II®; R and M Biometrics, Nashville, Tenn). This instrument was used for counting the number of attached parasites (all endogenous stages of *Cryptosporidium* in-

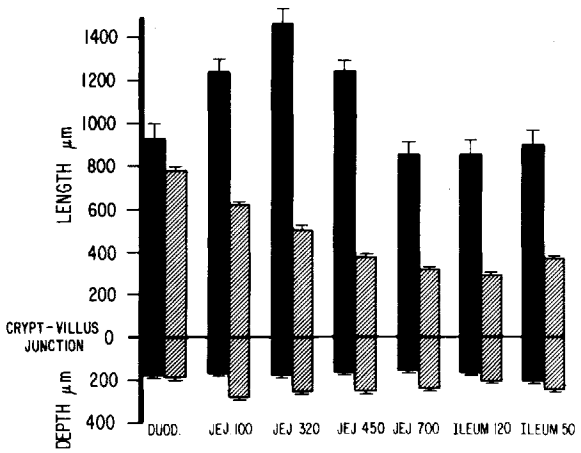

Figure 1. Length of villi and depth of crypts in the small intestines of two gnotobiotic calves 120 hr after oral inoculation with *Cryptosporidium* (slashed bars). Values for two control gnotobiotic calves are represented by solid bars. Each bar represents the mean value for 30–40 villi or crypts per site in each calf; brackets enclose the SE. Duod. = duodenum; jej. = jejunum. Numbers after site designations indicate centimeters distal to the duodenal site (jejunum) or proximal to the ileocecal valve (ileum).

cluded) along segments of epithelium (villous epithelium in the small intestine, crypt and surface epithelium in the large intestine) and for determination of the lengths of the epithelial segments selected.

Results

Clinical findings. Calves no. 1 and 2, which were inoculated with oocysts of *Cryptosporidium*, had a decreased appetite, lost strength, and became depressed beginning 72 and 48 hr after inoculation, respectively. Their feces subsequently became watery with clumps of mucus, and its color changed from brown to yellow-beige. Fecal dry weights were initially 37% and 18% for calves no. 1 and 2, respectively; these values fell to 8% at 60 hr and 5% at 96 hr and remained below 13% in both calves until the experiment was terminated at 120 hr. The body temperature of calf no. 2 increased by 1 C (to 40.4 C) at 72 hr; the body temperatures of calf no. 1 and the two calves given the control inoculum remained at 38.4 C–39.5 C throughout the experiment.

No clinical signs developed in control calves. Their feces remained formed, semisolid, and brown throughout the experiment. The initial dry weight of their feces was 38%, and this value gradually decreased to levels of 13% and 16%, respectively, by the end of the experiment. Neither infected nor control animals underwent marked changes in any of the cellular or chemical hematologic parameters evaluated.

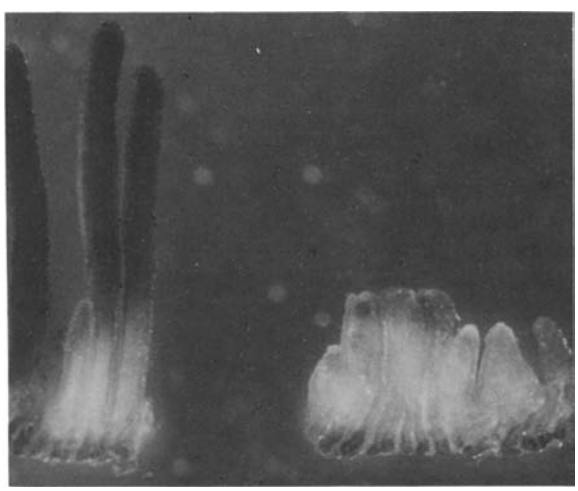

Figure 2. Whole-mount preparations dissected from the ileal mucosae of control (*left*) and *Cryptosporidium*-infected (*right*) gnotobiotic calves and viewed through a dissecting microscope. Villi are atrophic and crypts elongated in the preparation from the infected calf. The white spots in the background represent bubbles in the water and tissue debris.

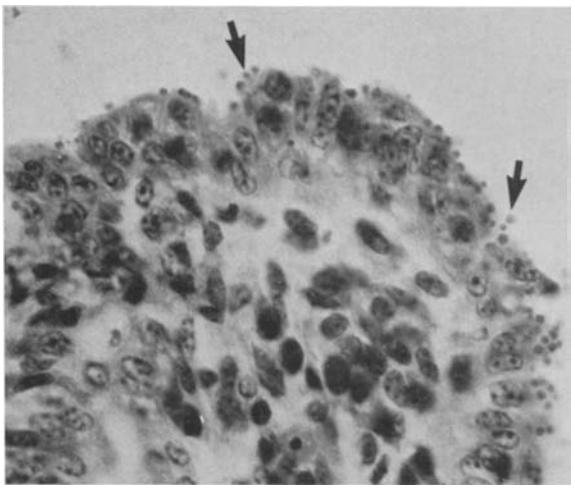

Figure 3. Photomicrograph of the tip of a villus in a histological section from the ileum of a gnotobiotic calf infected with *Cryptosporidium* (arrows). The epithelial surface is irregular. Epithelial cells are low columnar to cuboidal, and their nuclei are not aligned. A cellular infiltrate is seen in the lamina propria.

Parasitological findings. Cryptosporidial oocysts were first detected in the feces of calf no. 1 at 72 hr after inoculation and in the feces of calf no. 2 at 48 hr. Both calves continued to shed *Cryptosporidium* in their feces until the experiment was terminated. At necropsy, the parasites were associated with villous epithelium in the small intestine as well as with surface and crypt epithelium in the large intestine. The greatest numbers of parasites per unit (length) of epithelium tended to be in the lower small intestine (table 1). *Cryptosporidium* was not detected in any fecal sample or histological section from the control calves.

Pathological findings. No histological or ultrastructural lesions were found in intestinal sections from control calves. In contrast, the villi throughout the jejunum and ileum of infected calves were significantly shorter than those in the jejunum and ileum of control animals ($P < .01$; figures 1 and 2). Crypts from the jejunum and the

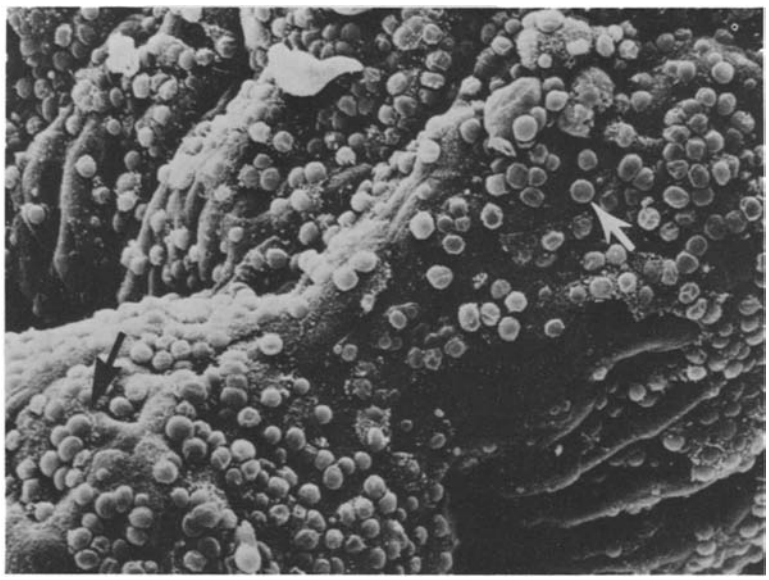

Figure 4. Scanning electron micrograph of the tips of two fused, atrophic villi from the ileum of a *Cryptosporidium*-infected gnotobiotic calf. Numerous parasites (arrows) protrude from the epithelium on the villi and the bridge (center) connecting them.

ileum 120 (i.e., the ileum 120 cm proximal to the ileocecal valve) of infected calves were significantly deeper than those from the same sites in controls ($P < .01$). The epithelium covering the atrophic villi in histological sections from infected calves was basophilic and usually low columnar to cuboidal, with disordered nuclei and an irregular surface (figure 3). In contrast, the crypt epithelium from the small intestine of infected calves remained tall and columnar but contained more mitotic figures than that from controls. The villous epithelium and lamina propria in some sections were infiltrated with a variety of mononuclear and polymorphonuclear inflammatory cells. Many villi from the infected calves were fused together. This fusion was apparent either as synechiae formed be-

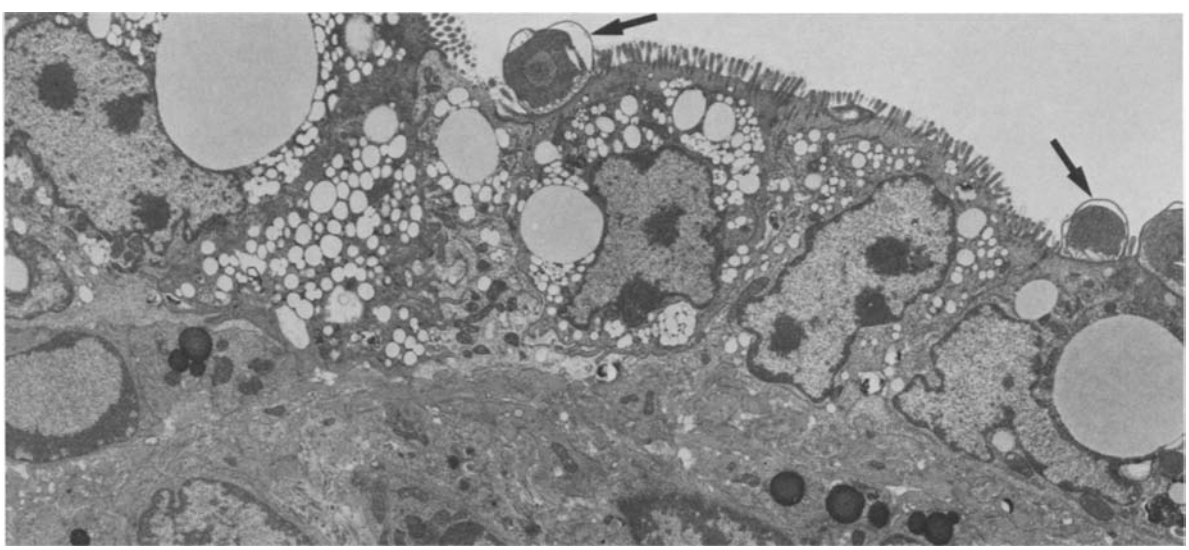

Figure 5. Transmission electron micrograph of epithelium from an atrophic villus from the ileum of a gnotobiotic calf infected with *Cryptosporidium* (arrows). The epithelial cells are low columnar to cuboidal and have short, sparse microvilli and extensive cytoplasmic vacuolation. Portions of two eosinophils can be seen beneath the epithelium; large electron-dense granules are evident at both the left and the right.

tween the epithelium of adjacent villi or as villi with an increased width of the lamina propria and with multiple lacteals and central veins. In the large intestine some foci of parasitized epithelium consisted of low columnar to cuboidal cells with nonaligned nuclei and irregular surfaces.

The fusion of some atrophic villi in infected calves was confirmed by scanning electron microscopy (figure 4). Transmission electron microscopy of epithelium from atrophic villi revealed parasites in various endogenous stages (figures 5 and 6). Villous absorptive cells frequently had cytoplasmic vacuolation (figures 5 and 6). Vacuolation was particularly marked in the ileum (figure 5). Occasional epithelial cells had electron-dense cytoplasm and nucleus (figure 6). These "dark" cells were interpreted to be degenerate and shrunken absorptive or goblet cells. Absorptive cell microvilli were irregular in length. They were short and sparse on many cells (figure 5); however, those immediately adjacent to *Cryptosporidium* were frequently longer than those further away from the parasite on the same or other absorptive cells (figure 6). Occasionally, parasitized epithelial cells were observed, with disrupted apical plasma membranes and cytoplasmic protrusion into the intestinal lumen (figure 6).

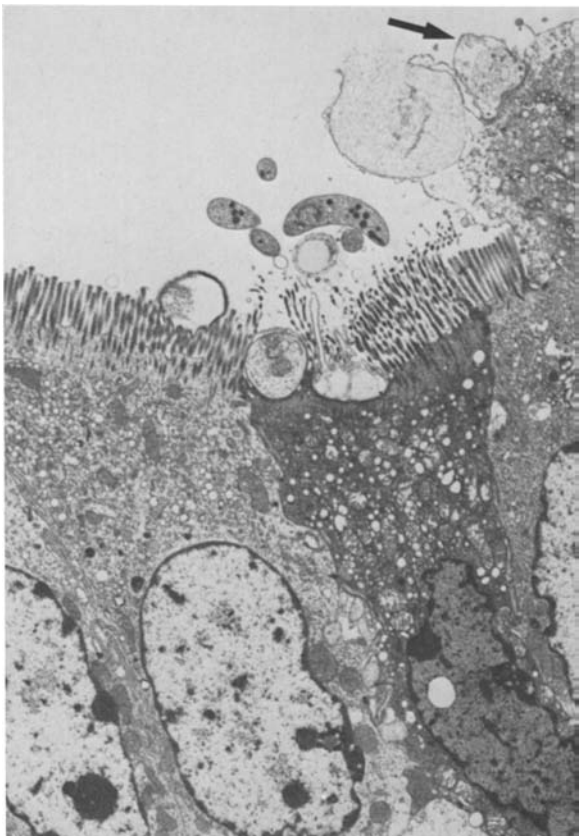

Figure 6. Transmission electron micrograph of villous epithelium from the jejunum of a *Cryptosporidium*-infected gnotobiotic calf. Microvilli are irregular in length, and epithelial cell cytoplasm is finely vacuolated. Several merozoites (crescentic, with electron-dense granules) have been released into the intestinal lumen at the center of the photograph. The residual body of the schizont and the parasitophorus envelope remain attached to a shrunken, electron-dense epithelial cell. The epithelial cell on the right has a disrupted apical plasma membrane with no visible microvilli, and *Cryptosporidium* (arrow) is attached to a portion of the cell that is protruding into the lumen.

Discussion

Gnotobiotic calves inoculated with oocysts of *Cryptosporidium* that had been treated with potassium dichromate and peracetic acid became infected with *Cryptosporidium* and developed clinical signs and enteric lesions. In contrast, control gnotobiotic calves remained normal after the inoculation of similar material that had been passed through a filter for the removal of oocysts (but not of any putative viruses). We were unable to cultivate extraneous agents from the inocula or from the feces of any of the calves. We were also unable to demonstrate infectious agents other than *Cryptosporidium* by direct light and electron microscopic examination of feces from the calves or by transmission electron microscopic study of sections of intestine from the animals. These results provide strong evidence that the calves inoculated with oocysts were monoinfected with *Cryptosporidium* and that *Cryptosporidium* caused their enteric lesions and diarrhea. The data tend to confirm and extend a previous report that *Cryptosporidium* is pathogenic in monoinfected gnotobiotic animals [18].

Both the enteric lesions (characterized by damage to and loss of epithelial cells, villous atrophy and crypt hyperplasia, and infiltration with a mixture of inflammatory cells) and the signs (diarrhea, depression, and anorexia) were similar to those associated with experimental and naturally acquired cryptosporidial infections in conventional animals [7, 9, 10] and people (10–17]. In aggregate, the evidence strongly suggests that *Cryptosporidium* causes enteric disease during naturally acquired in-

fection. Subclinical infections are common. Variations in signs and lesions associated with the infection are probably caused in part by variations in the dose (and possibly the virulence) of the *Cryptosporidium* ingested. Calves can shed as many as 10^8 cryptosporidial oocysts/ml of feces [4]. We assume that calves sometimes naturally encounter doses of oocysts as large as that used here (10^8) because it seems reasonable that calves reared under conditions of poor hygiene could ingest 1 ml of feces. There is also considerble variation in signs and lesions that is dependent on the species, age, and immune status of the host. In our experience, experimentally infected newborn calves usually develop diarrhea [7], but experimentally infected mice do not [25]. Presumably, the amount of mucosal damage is less or the compensatory capacity of the undamaged mucosa is greater in newborn mice than in calves. Adult mice are comparatively resistant to the infection, but immunodeficient (nude) newborn mice developed persistent infection and diarrhea, and some die [25]. Calves that recover from experimental cryptosporidial infection are resistant to a second challenge with the organism (author's unpublished data).

We suspect that diarrhea occurs in infected animals because extensive damage to epithelial cells and villous atrophy result in malabsorption. The occurrence of hyperplastic crypt epithelium along with damaged villous epithelium and atrophic villi in infected calves represents evidence that the lesions develop as a result of accelerated destruction or loss (rather than decreased production) of epithelial cells. The mechanism(s) by which *Cryptosporidium* destroys or accelerates the loss of epithelial cells is (are) unknown. Some evidence suggests that villous atrophy can be induced by cell-mediated immune responses to protozoa and helminths [26, 27]. However, the occurrence of villous atrophy in *Cryptosporidium*-infected nude mice [25] suggests that, although T lymphocytes are required for the development of villous atrophy in some enteric protozoan infections, this is not the case in cryptosporidiosis. Perhaps *Cryptosporidium* damages epithelial cells directly through some toxic, metabolic, or physical effect.

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