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Experimental Cecitis in Gnotoxenic Chickens Monoassociated with *Clostridium butyricum* Strains Isolated from Patients with Neonatal Necrotizing Enterocolitis

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An animal model for *Clostridium butyricum* necrotizing cecitis has been developed in axenic chickens inoculated orally between 2 and 50 days of life. Cecitis was obtained with two *C. butyricum* strains isolated from neonatal necrotizing enterocolitis and not with a *Clostridium beijerinckii* strain from dairy products; the rate of colonization of the intestinal tract by this strain was lower than that obtained with *C. butyricum* strains. The clinical findings showed a slow gain in body weight. The cecitis lesions were well developed 3 and 4 weeks after oral inoculation, including enlargement with an increase of the cecum weight-body weight ratio, a marked hyperplasia, congestion, inflammatory infiltrate and pneumatosis of the cecal wall and mesentery, hemorrhage in the lamina propria and submucosa, and ulcerations and necrotic areas in the mucosa. By immunofluorescence and electron microscopy, the bacterial cells were located in the cecal lumen and in necrotic areas of the mucosa. The presence of 4% lactose in the diet seemed to be a prerequisite for the development of cecitis in chickens. A gradual rise of fluorescent antibodies in the sera was observed.

Howard et al. (6) reported the presence of *Clostridium butyricum* in the blood of 9 of 10 and in the stools of 6 of 10 newborns with neonatal necrotizing enterocolitis (NNE). The authors concluded that *C. butyricum* was probably the final step in the pathogenesis of NNE in this group of babies. Since this observation, *C. butyricum* was recovered from several outbreaks of NNE (10, 16, 25, 26) and also from fecal specimens of healthy babies (5, 25). Various microorganisms, including *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *Salmonella* spp., *Clostridium perfringens*, *Clostridium difficile*, coronavirus, rotavirus, and enterovirus, were also associated with NNE (8).

Attempts to induce NNE with *C. butyricum* in animals have not been successful. Lawrence et al. (12) reported hemorrhagic enteritis in neonatal monoxenic rats, but failed to reproduce this previous result (11).

In the cecum of gnotoxenic chickens monoassociated with various microorganisms, we observed that *C. butyricum* produced volatile fatty acids and that its establishment was ca. 10^8 cells per g of the content (29). The purpose of this study was to develop a experimental chicken model for *C. butyricum* cecitis wherein necrotic lesions and pneumatosis could be induced through oral inoculation with human strains.

MATERIALS AND METHODS

Bacterial strains and growth. Two *C. butyricum* strains were used: strain CB1002 isolated from an infant with a fatal case of NNE (16) and strain CB128 from feces of a newborn with NNE (P. Raibaud, Centre National de Recherches Zootechniques, Jouy en Josas, France). As a control, we used *C. beijerinckii* CNRZ 530 from dairy products (20). The bacteriological identification of these strains was previously described (14).

The bacteria were grown in TY broth (14) with 0.5% (TYG) or 6% (TYG6) (wt/vol) glucose. The spores were obtained in D medium (9). The media were incubated anaerobically for 18 h at 37°C.

For bacterial counting, we used a B′ agar medium (22) in tubes (8 by 400 mm) and a dilution medium (peptone broth) (17). The tubes were incubated aerobically for 18 h at 37°C.

Maintenance and inoculation of chickens. A Warren × albino strain was used. The axenic birds were obtained by a method already described (13). On the third day of life, cloacal droppings were collected for a sterility check. Three days later, the birds were transferred from the hatching isolator to several experimental isolators. One group remained axenic. The others were monoassociated with *C. butyricum* or *C. beijerinckii* per os with a few drops of culture containing 10^8 viable cells and 10^7 spores per ml. Sterility and bacterial establishment were checked in droppings 4 days later and then at weekly intervals.

The chickens were fed ad libitum a semisynthetic diet (Table 1) pelleted, packed in double-vacuum bags, and sterilized by 4 megarads of radiation (28). In one experiment, lactose was replaced by starch at the same concentration (40 g/kg).

Six hours after the beginning of the meal, the chickens were weighed and killed by intraventricular injection of 5% sodium pentobarbital (Abbott Laboratories, Chicago, Ill.) at various intervals after inoculation (chickens infected with strain CB1002) and at 4 weeks after inoculation (chickens infected with strains CB128 and CNRZ 530 and axenic chickens). Blood was drawn off by heart puncture, and the serum was stored at −20°C. Ceca were collected immediately after death and weighed. Cecal contents were removed for pH control and bacteriological examinations.

Bacterial count. As soon as the cecal samples (1 g) had been diluted, they were homogenized for 20 to 30 s with an Ultra-Turrax (Osi, Paris, France) and diluted in a dilution

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TABLE 1. Chicken diet

Ingredients (g/kg)	Diet L	Diet O
Fish meal	230	230
Maize starch	669	709
Maize oil	40	40
Cellulose (colmacil)	20	20
Salt mixture[a]	7	7
Vitamin mixture[b]	5	5
Lactose	40	0

[a] Amounts supplied (g/kg of diet): $MgSO_4 \cdot 7H_2O$, 6; $MnSO_4$; Fe citrate, 0.40; $ZnCl_2$, 0.0098; Na_2SiO_3, 0.06; KI, 0.048; $NaBO_2$, 0.030; $Na_2MoO_4 \cdot 2H_2O$, 0.024; $KAl(SO_4)_2$, 0.012; H_3BO_3, 0.0096; $CuSO_4$, 0.0096; $CoSO_4 \cdot 7H_2O$, 0.0024; Na_2SeO_3, 0.0001.

[b] Amounts supplied (g/kg of diet): retinol acetate (165 mg/g), 0.02; cholecalciferol (2.5 mg/g), 0.02; α-tocopherol (250 mg/g), 0.12; thiamine, 0.0118; riboflavin, 0.0176; pyridoxine, 0.0176; nicotinic acid, 0.17; folic acid, 0.035; p-aminobenzoic acid, 0.294; inositol, 0.294; Ca pantothenate, 0.0588, choline chlorhydrate (0.25 mg/g), 4; ascorbic acid, 0.294.

medium; 0.1 ml of 10-fold serial dilutions was poured into B′ agar medium.

Histological technique. Samples of the gastrointestinal tract were fixed in Bouin solution, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin. In some cases, Gram staining was used for detection of clostridia.

Preparation of antiserum. *C. butyricum* CB1002 vegetative cells were grown in TYG6, washed with 0.01 M phosphate–0.15 M NaCl buffer (phosphate-buffered saline [PBS]) (pH 7.2), and suspended in PBS (10^8 cells per ml). New Zealand rabbits weighing 1.5 to 2 kg were inoculated intravenously with 1 ml of cell suspension once a week for 3 weeks, followed by subcutaneous injection of 1 ml of cell suspension with 1 ml of complete Freund adjuvant in several sites. Two weeks after the last injection, the rabbits received 1 ml of bacterial suspension intravenously and were bled 10 days later.

Histological examination by indirect immunofluorescence. Slides of acetone-fixed chicken cecal sections were incubated with a dilution of antiserum against strain CB1002. Fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulins were used as a second antibody. Preparations were observed with a Zeiss epifluorescent UV microscope.

Serological technique. Indirect immunofluorescence was performed as previously described (7) with the strain used for chicken inoculation. The bacteria were grown anaerobically in TYG6 for 18 h, washed twofold in PBS, and fixed on a glass slide with methanol for 10 min. Twofold dilutions of chicken sera in PBS were incubated with fixed bacteria for 30 min at room temperature. Fluorescein isothiocyanate-conjugated anti-chicken immunoglobulins (Nordic Immunological Laboratories, Tebu, France) were used at a 1:50 dilution in PBS for 30 min at room temperature.

Electron microscopy. Sections of cecum were fixed with 2.5% glutaraldehyde in cacodylate buffer, postfixed in 1% OsO_4, embedded in Epon, and stained with uranyl acetate and lead citrate (18).

Presentation of data and statistical methods. The data are presented as the mean ± standard error of the mean and were evaluated statistically by Student's *t* test.

RESULTS

Clinical findings. In all experiments, no death, acute illness, or diarrhea was recorded in infected chickens during the time course of observation (4 weeks maximum), in spite of the level of 10^7 CFU of *C. butyricum* strains per g in feces as early as day 4 postinoculation. However, the infected chickens showed a significantly slower weight gain compared with the control group. In two different experiments, pooling 12 axenic and 7 monoassociated chickens, the slaughter weight was 477 ± 16 and 351 ± 32 g, respectively ($P < 0.001$).

Postmortem findings. (i) Macroscopic cecal observations. The kinetics of the onset of cecal changes were shown in chickens monoassociated with *C. butyricum* CB1002 (Table 2). At 1 and 2 weeks after inoculation, the cecum contained only the fluid. Severe changes were observed at week 3, and especially at week 4, after inoculation, i.e., absence or small amounts of intracecal gas, hemorrhagic content and necrotic debris in the lumen, ulcerative hemorrhages of the mucosa, enlargement of the cecal wall, enlargement and sclerosis of the mesentery, and in some cases the presence of gas cysts (Fig. 1). Cecal changes with time were similar when 2- to 50-day-old axenic chickens were inoculated. No cecal changes were seen 4 weeks after oral inoculation in two 50-day-old chickens.

Four weeks after inoculation, *C. butyricum* CB128 produced cecal changes similar to those observed with CB1002,

TABLE 2. Cecal changes observed in chickens nomoassociated with *C. butyricum* or *C. beijerinckii*

Strain	Weeks after initial infection (no. of animals)[a]	Concn of lactose in the diet (%)	Cecum weight-body weight ratio ($\times 10^{-3}$) (mean ± SEM)	Log_{10} of viable *Clostridium* cells per g of cecal content[b]	pH of cecal content (mean ± SEM)	Macroscopic and histological findings[c] (no. of animals)
Control	6 (11)	4	3.37 ± 0.47		7.01 ± 0.40	N(11)
C. butyricum CB1002	1 (3)	4	3.79 ± 0.68[d]	8.4 (8.0–8.8)	6.16 ± 0.24[e]	N(2); A(1)
	2 (3)	4	7.30 ± 0.88[d]	7.9 (7.5–8.1)	6.40 ± 0.18[f]	A(1); B(2)
	3 (3)	4	5.76 ± 0.73[d]	8.2 (8.0–8.6)	5.90 ± 0.59[e]	A(1); B(2)
	4 (4)	4	5.63 ± 1.70[e]	8.2 (7.9–8.7)	6.40 ± 0.29[f]	A(1); B(1); C(2)
	4 (4)	0	4.27 ± 1.05	8.3 (7.8–8.5)	6.60 ± 0.07	N(4)
C. butyricum CB128	4 (4)	4	8.50 ± 5.24[e]	8.3 (7.5–8.9)	6.26 ± 0.36[e]	A(1); B(1); C(2)
C. beijerinckii CNRZ 530	4 (4)	4	4.15 ± 1.20	4.1 (3.7–4.4)	7.13 ± 0.14	N(4)

[a] The oral inoculation was made in 7-day-old chickens.

[b] The spore count estimated by heating the cecal content at 70°C for 10 min ranged from 1×10^4 to 5×10^4/g. Shown is the mean (range).

[c] N, Normal; A, presence of a high amount of gas, moderate inflammation of the mesentery, moderate hyperplasia, and congestion of the submucosa and serosa; B, presence of a small amount of gas, reduction of fluid content volume, enlargement of the wall and the mesentery, hyperplasia, congestion, and inflammatory infiltrate of the mucosa, submucosa, musculosa, and serosa; C, no gas, a small amount of hemorrhagic content, hemorrhagic ulcerations of the mucosa, marked enlargement, congestion, inflammatory infiltrate and pneumatosis of the mucosa, submucosa, musculosa, and serosa, patchy hemorrhages in the lamina propria and submucosa, necrotic areas of the mucosa.

[d] Significantly different than control; $P < 0.001$.

[e] Significantly different than control; $P < 0.01$.

[f] Significantly different than control; $P < 0.02$.

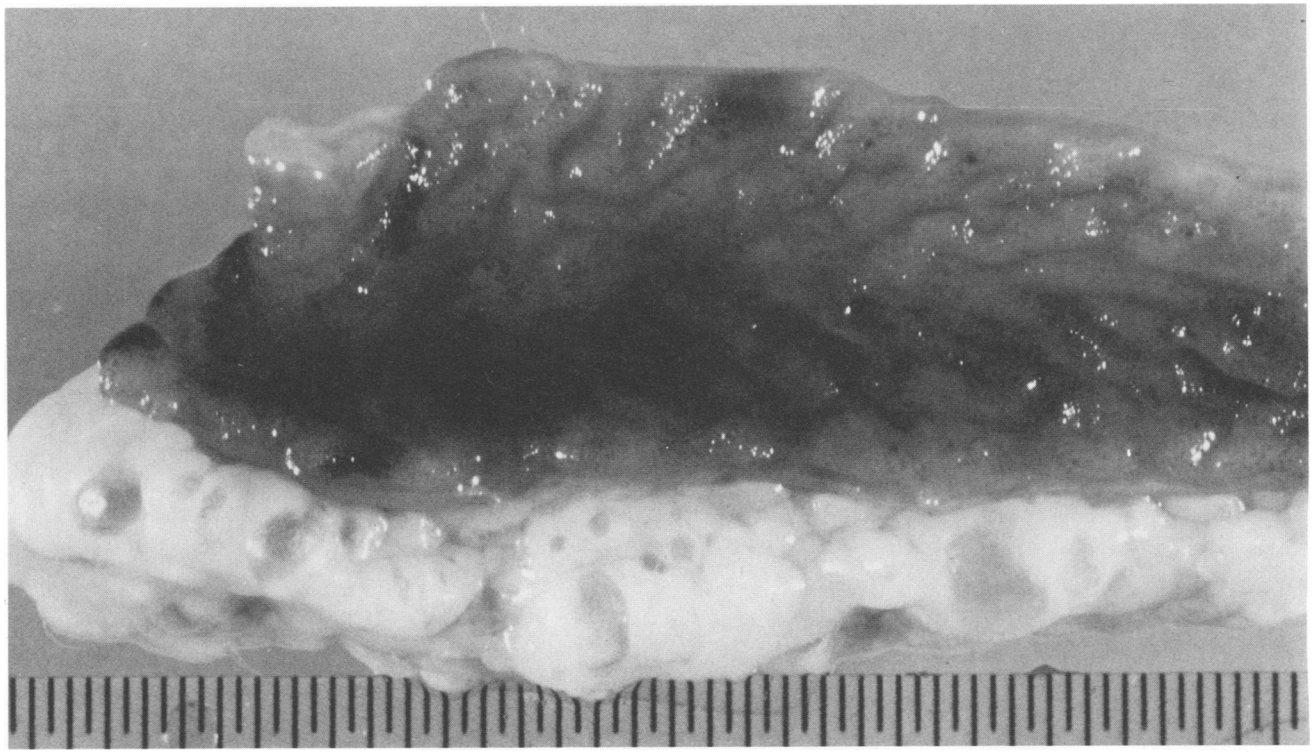

FIG. 1. Macroscopical aspect of chicken cecum opened longitudinally. Monoassociation with *C. butyricum* CB1002 (4 weeks after infection) and 4% lactose diet. Note the ulcerative hemorrhage of the mucosa and gas cysts in the mesentery (distance between two lines, 0.5 mm).

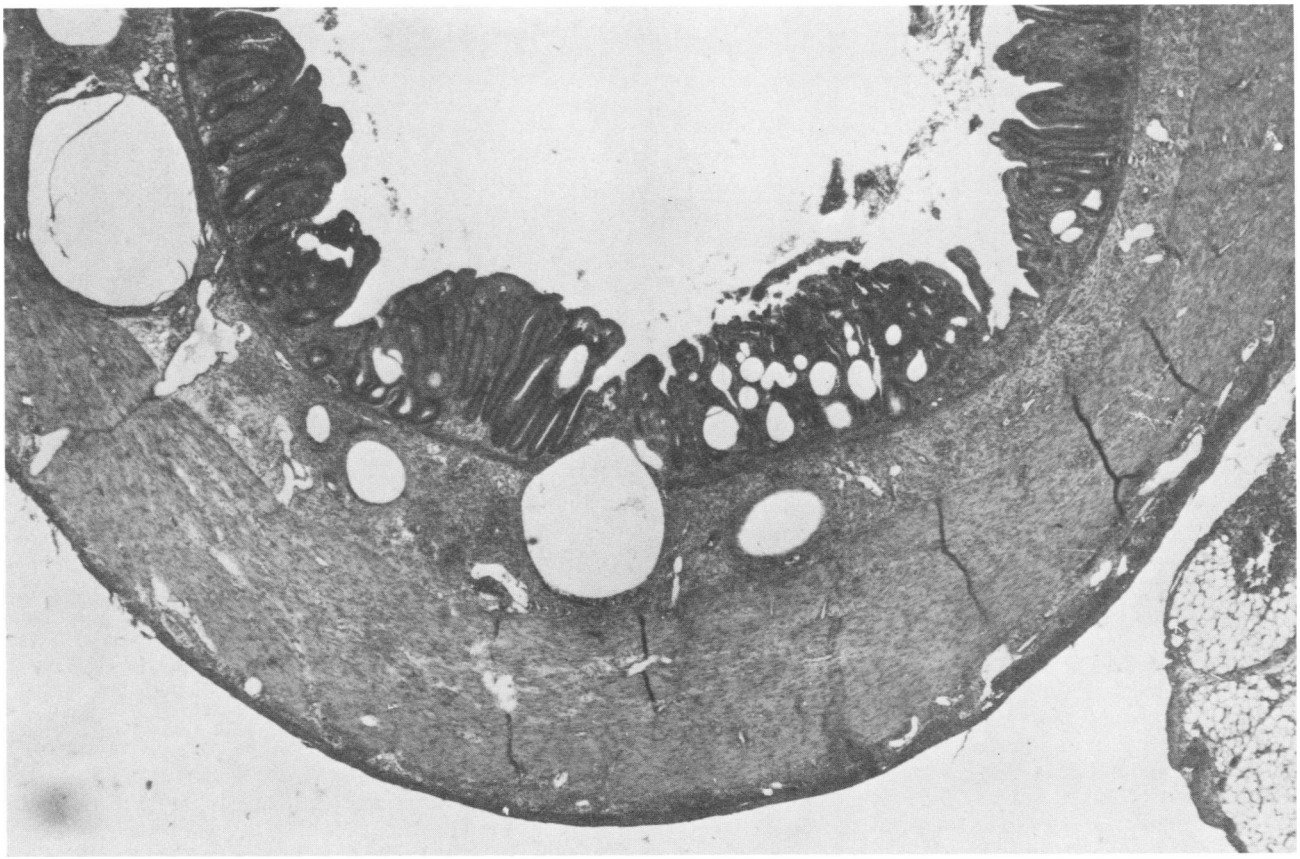

FIG. 2. Histological section of chicken cecum. Monoassociation with *C. butyricum* CB1002 and 4% lactose diet. At 4 weeks after inoculation, the cecum showed enlargement and congestion of the mucosa, submucosa, and musculosa and inflammatory infiltrate and gas cysts in the mucosa and submucosa (hematoxylin and eosin stain; ×25).

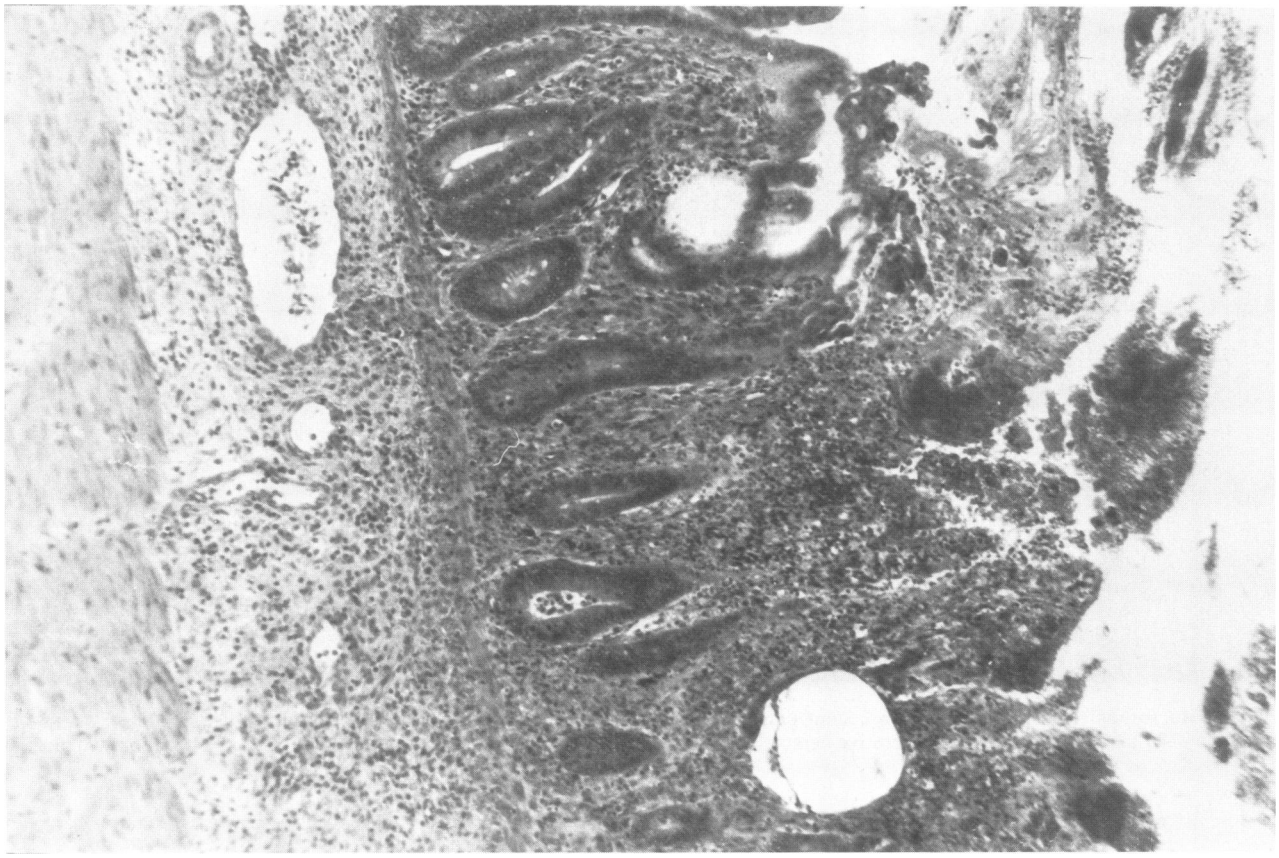

FIG. 3. Histological section of chicken cecum. Monoassociation with *C. butyricum* CB1002 and 4% lactose diet. Note the severe ulcerative necrosis of the mucosa, gas cysts in the mucosa and submucosa, congestion and inflammatory infiltrate in the lamina propria, and congestion of the submucosa (hematoxylin and eosin stain; ×100).

but inoculation with *C. beijerinckii* did not lead to cecal changes.

(ii) Cecal pH and bacterial count. The pH of the cecal content in chickens fed with the diet containing 4% lactose and monoassociated with *C. butyricum* CB1002 and CB128 was lower than that of their axenic counterparts and that of the chickens monoassociated with *C. beijerinckii* CNRZ 530. The rate of colonization by *C. butyricum* strains was higher than that by the *C. beijerinckii* strain ($P < 0.001$). In chickens monoassociated with *C. butyricum* CB1002, the pH was low and the rate of colonization was high from 1 week after inoculation.

(iii) Cecum weight-body weight ratio. The cecum weight-body weight ratio increased in chickens monoassociated with *C. butyricum* CB1002 from week 2 after inoculation and with CB128 from week 4 after inoculation compared with uninfected animals and chickens monoassociated with *C. beijerinckii* (Table 2).

Histological findings. Light microscopy and electron microscopic examinations showed marked histological changes between 1 and 4 weeks after inoculation.

At 1 and 2 weeks after inoculation with *C. butyricum* CB1002, moderate enlargement and congestion of the cecal submucosa was observed. The bacteria were found in the cecal lumen, and the bacterial cells did not specifically attach to the microvilli of the brush border.

At 3 and 4 weeks after inoculation with *C. butyricum* CB1002 and at 4 weeks after inoculation with *C. butyricum* CB128, the thickness of the mucosa, submucosa, and muscularis markedly increased. Inflammatory infiltrate, congestion, and hemorrhages were seen in the lamina propria and submucosa. The most prominent lesions were characterized by pneumatosis cysts of various sizes in the three layers of the cecal wall and necrotic areas of the mucosa and submucosa (Fig. 2 and 3). Gram staining, immunofluorescence, and electron microscopy showed whole and disrupted bacteria in the necrotic areas of the mucosa, but none in the gas cysts. Large intercellular spaces between the epithelial cells were observed by electron microscopy before the detachment and disintegration of the epithelial cells (Fig. 4). The other segments of the digestive tract did not exhibit any modification.

Influence of alimentation. When the chickens monoassociated with *C. butyricum* CB1002 were fed with the basal lactose-free diet, neither macroscopic nor microscopic changes were observed in the cecum 4 weeks after inoculation, despite the establishment of *C. butyricum* at a level similar to that in chickens fed a diet containing 4% lactose ($P > 0.5$). The pH of the cecal content was decreased, but the cecum weight-body weight ratio was unchanged with regard to the control axenic chickens (Table 2).

Serological response to infection. In the indirect fluorescent antibody assay with homologous cells as the source of antigen, a rise of fluorescent antibody was observed in sera of chickens monoassociated with *C. butyricum* CB1002 from week 2 (reciprocal mean titer, 10 ± 5) to week 4 (48 ± 18) (Fig. 6). The chickens monoassociated with *C. butyricum* CB128 and those monoassociated with *C. butyricum* CB1002

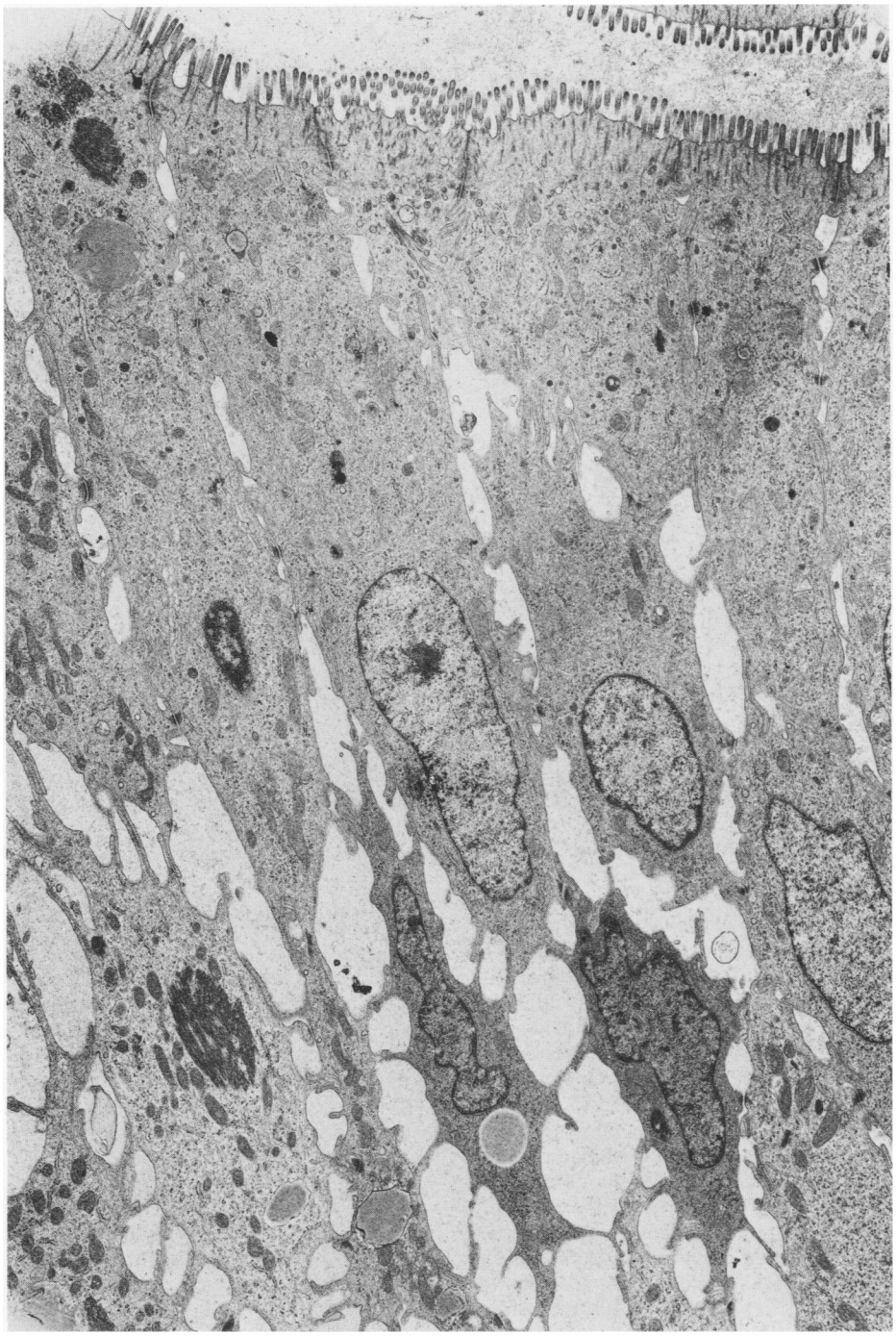

FIG. 4. Electron microscopy of chicken cecum. Monoassociation with *C. butyricum* CB1002 and 4% lactose diet. The epithelial cells show an intact brush border and intercellular spaces mainly at the basal pole (×3,000).

and receiving a lactose-free diet showed an antibody response (28 ± 24 and 14 ± 4, respectively) at week 4, but with *C. beijerinkii* CNRZ 530 the level of fluorescent antibody was lower (1.7 ± 0.5; $P < 0.001$). In five control axenic chickens, no fluorescent antibodies were detected with these three clostridia strains as antigens.

DISCUSSION

These experiments demonstrate that *C. butyricum* CB1002 and CB128 can induce cecitis in axenic chickens. The macroscopic pathological findings consisted of enlargement of the cecum, the presence of hemorrhagic fluid in the lumen, and numerous ulcerations of the mucosa. Microscopic findings were hyperplasia and pneumatosis of the cecal wall, necrotic areas, congestion, hemorrhage, and inflammatory infiltrate in the mucosa and submucosa. These findings were similar to those observed in human newborns with NNE (1, 23, 27).

However, in human newborns the illness was often fulminant and dramatic, and the lesions were located in the ileum

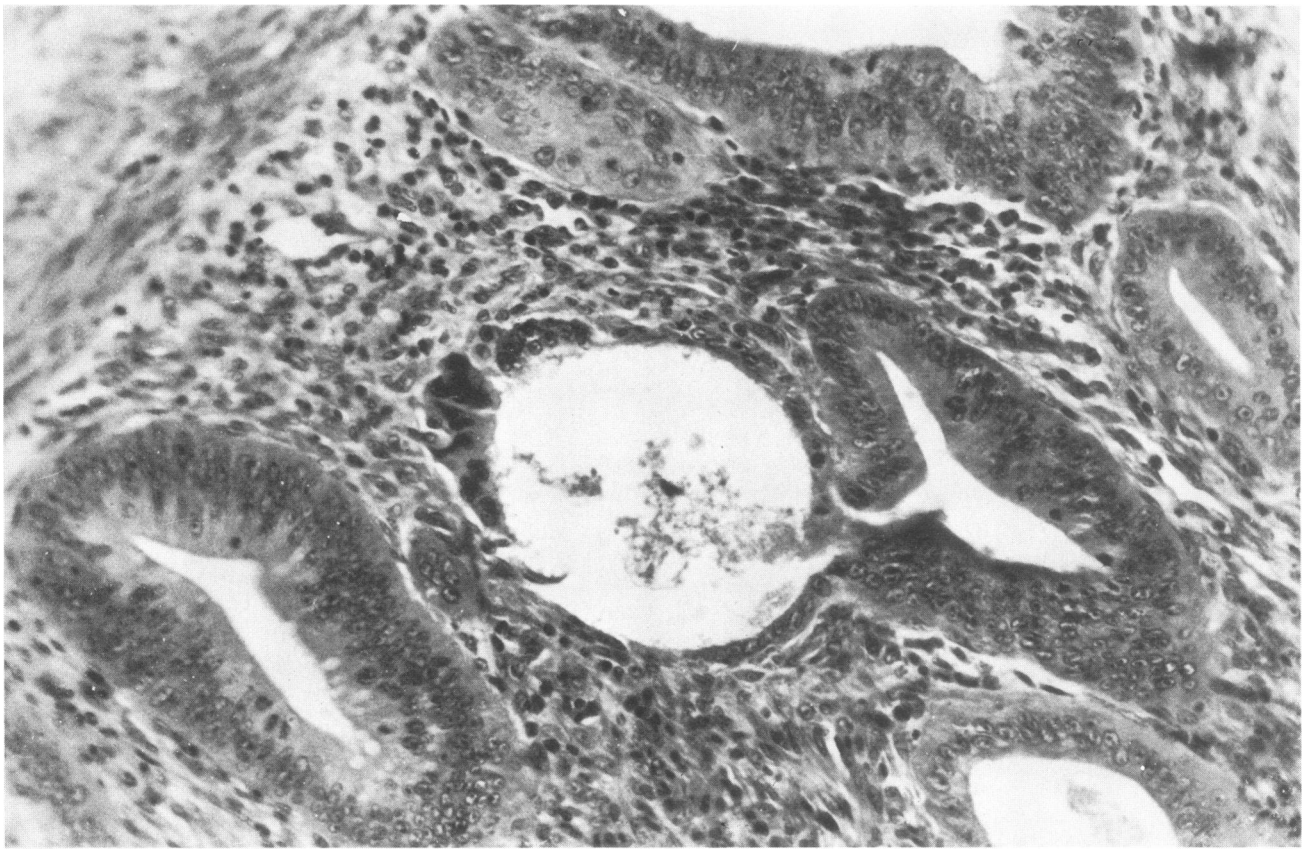

FIG. 5. Gas cysts in the lamina propria connected to a crypt epithelium. No bacteria are seen in the gas cyst (hematoxylin and eosin stain; ×250).

and the colon (23), whereas the chickens showed a chronic disease, leading a slower body weight gain, and the lesions were only observed in the cecum. Moreover, the populations levels of *C. butyricum* in monoassociated chickens were higher than those in the human newborns (25). Host susceptibility to this strain might explain the differences in the disease observed in natural conditions and in the chicken model.

Pneumatosis cystoides intestinalis (PCI) is often encountered in NNE (2, 15). It has been suggested that the gas cysts are formed by gas-producing bacteria (3, 33). Yale et al. (31–33) produced PCI in germ-free rats by injecting *C. perfringens* culture into an isolated segment of strangulated intestine, the wall of the terminal ileum and cecum, or the peritoneal cavity. PCI was obtained also with *C. tertium* (30). But these authors failed to produce PCI in germ-free rats monoassociated with *C. perfringens* without surgical procedure (32). In our experiments, numerous gas cysts were observed in the cecal wall of chickens monoassociated with *C. butyricum*. The gas cysts were seen from week 3 after inoculation in the cecum wall and also in some cases in the mesentery. No bacteria were observed in the gas cysts by Gram staining or by indirect immunofluorescence. The pathogenesis of the gas cysts probably consists of an accumulation of gas produced by bacteria in the cecal lumen through rupture of epithelium integrity (Fig. 5) or through spaces between epithelial cells (Fig. 4).

The *C. butyricum* cells were mainly located in the cecal lumen or in the crypts. Whole and disrupted bacteria were seen only in the necrotic areas of the cecal wall, suggesting that a soluble factor could be involved in the pathogenesis of *C. butyricum*. It has been shown that butyrate has cytostatic and cytotoxic effects on cultured cells (4) and could be an important toxic component in gingival inflammation in humans (24). In the cecum of chickens monoassociated with *C. butyricum* and fed a diet containing 4% lactose, volatile fatty acid production was 9.9 ± 3.1 mm/kg (44% acetate and 56% butyrate) (29). When the lactose was omitted, only traces of volatile fatty acid were detected (unpublished data), and cecal changes did not occur, despite bacterial colonization in a range similar to that for chickens receiving the diet with 4% lactose. The amount of fermentable carbohydrates which can reach the cecum has an effect on the amount of volatile fatty acid produced by clostridia and could also induce the synthesis of a toxic factor.

C. butyricum CB1002 and CB128 isolated from NNE colonized the digestive tract at high levels (10^7 to 10^8 CFU/g of cecal content) and produced similar cecal lesions in axenic chickens. Serum fluorescent antibodies were detected from week 2 after inoculation and rose gradually until week 4 after inoculation. The onset of cecal lesions and the gradual rise of fluorescent antibodies after infection suggest that these antibodies did not have protective effects. *C. beijerinckii* CNRZ 530 isolated from milk did not cause pathological changes, but the implantation of this strain did not exceed 2×10^5CFU/g of cecal content, and the antibody titers were lower. Further investigations with other *C. butyricum* strains are in progress.

Birds are not common laboratory animals to use when studying human infections. Newborn conventional chickens

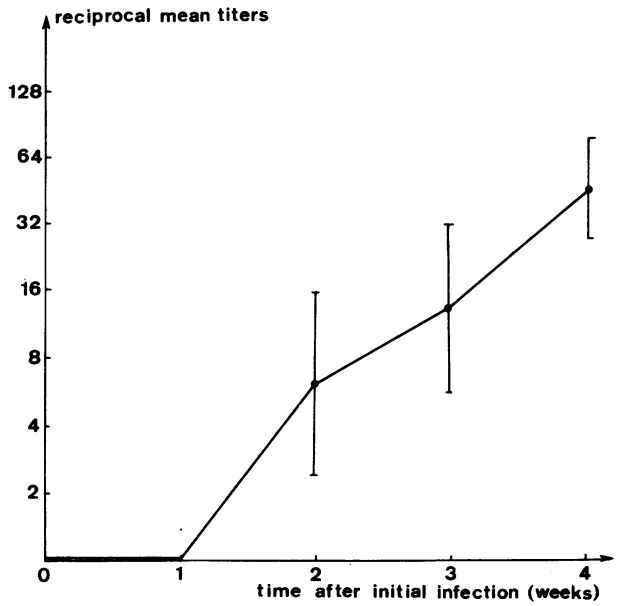

FIG. 6. Reciprocal mean serum antibody titers by indirect fluorescent antibody assay in gnotoxenic chickens fed a diet containing lactose and monoassociated with *C. butyricum* CB1002 (●). Means ± standard errors of the mean are shown by bars.

were previously used as an experimental model for diarrhea induced by *Campylobacter* spp. (21), and quails have been used for cancer research (19). Our work shows that 2- to 50-day-old axenic chickens are a suitable experimental model for an enteropathogenic study of *C. butyricum* because the main histological lesions of NNE (congestion, hemorrhage, necrosis, and pneumatosis) were obtained.

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