Experimental Production of Hemorrhagic Enterotoxemia by *Clostridium perfringens* Type C in Maturing Lambs

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ABSTRACT

Maturing lambs, eight to nine months old, were dosed by the intraduodenal route with various preparations of *Clostridium perfringens* type C. Whole cultures of this organism or cells suspended in fresh medium, both supplemented with soybean flour as a protease inhibitor, produced acute fatal hemorrhagic enterotoxemia in these animals. The latter preparation was more effective than the former in causing disease. Without the soybean supplement the inocula did not produce fatal disease. Dosing with toxic cell-free culture supernatant fluid, with or without soybean supplement, had no lethal effect. Animals that died showed severe hemorrhagic enteritis with necrosis and sloughing of the mucosal epithelium, involving jejunum, ileum and part of duodenum. These lesions were similar to those seen in natural cases of hemorrhagic enterotoxemia in neonatal animals. This experiment demonstrated that nonimmune animals are normally protected against *C. perfringens* type C enterotoxemia by adequate levels of pancreatic proteases in the intestine, and that factors which inhibit or reduce these enzymes predispose animals for the development of this disease.

Key words: *Clostridium perfringens,* beta toxin, hemorrhagic enterotoxemia, necrotic enteritis.

RÉSUMÉ

Cette expérience consistait à introduire dans le duodénum d'agneaux âgés de huit à neuf mois diverses préparations de *Clostridium perfringens* du type C. Des clostridies, sous la forme de cultures complètes ou de suspensions dans un milieu de culture frais, auxquelles on ajouta de la farine de fève de soya, pour inhibiber les protéases pancréatiques, produisirent une entérotoxémie aiguë et fatale, chez ces agneaux. la dernière préparation se révéla plus efficace que la première. Sans la farine de fève de soya, les inoculums ne produisirent pas une maladie fatale. L'introduction dans le duodénum d'un surnageant toxique de culture, débarrassé des clostridies, avec ou sans farine de fève de soya, n'exerça aucun effet mortel. Les agneaux qui moururent développèrent une entérite hémorragique marquée qui s'accompagnait d'une desquamation et d'une nécrose de l'épithélium de la muqueuse du jéjunum, de l'iléon et d'une partie du duodénum. Ces lésions ressemblaient à celles qu'on retrouve dans les cas naturels d'entérotoxémie hémorragique, chez les animaux nouveau-nés. L'expérience démontra que les animaux non immunisés sont normalement protégés contre l'entérotoxémie précitée par un taux intestinal adéquat de protéases pancréatiques et que les facteurs responsables de l'inhibition ou de la diminution de ces enzymes prédisposent les animaux au développement de cette condition.

Mots clés : *Clostridium perfringens,* toxine bêta, entérotoxémie hémorragique, entérite nécrotique.

INTRODUCTION

Hemorrhagic enterotoxemia or necrotic enteritis caused by *Clostridium perfringens* type C is an acute, usually fatal disease of very young animals. It has been reported in newborn piglets, calves, lambs and foals (1-7). The disease also occurs in humans, particularly in children less than nine years old, in Papua New Guinea, and has been reported occasionally in certain other countries (8). The major factor involved in the pathogenesis of this disease is beta toxin which is produced by this clostridium in the intestinal tract under favourable conditions of growth.

The disease has been produced experimentally, with varying success, in young suckling pigs by feeding *C. perfringens* type C cultures and toxins (9). A successful infection of a young dairy calf has also been reported (3). Older animals appear to be refractory to experimental infection with this organism. The distinct age incidence could not be explained on the basis of acquired specific immunity alone; thus other factors have been considered. Griner (10) postulated that trypsin played an important role in the age incidence of this disease. He suggested that animals normally may be protected by trypsin in the intestine at levels which destroy the sensitive beta toxin, but that newborn animals may have either a deficiency of trypsin or they may acquire trypsin inhibitors in the colostrum. In an experiment to prove the importance of trypsin deficiency in human necrotic enteritis, Lawrence and Cooke (11) showed that a persistent low protein diet combined with dietary protease inhibitors in the presence of *C. perfringens* type C could cause necrotic enteritis in guinea pigs.

To determine whether similar factors are involved in the pathogenesis of *C. perfringens* type C enterotoxemia in domestic animals an experiment was conducted on weaned, maturing lambs using various *C.*

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perfringens preparations with and without a trypsin inhibitor.

MATERIALS AND METHODS

ANIMALS

Dorset lambs, eight to nine months old, weighing 41 to 54 kg, were used. These animals originated from our Institute's healthy flock and had never been vaccinated or treated for any disease. Each animal was fitted with an indwelling intraduodenal cannula and was accommodated in a special facility as described previously (12). Guidelines given in "Guide to the Care and Use of Experimental Animals" (Canadian Council on Animal Care, Ottawa, Ontario) were followed.

INOCULA

Clostridium perfringens type C, isolated from a case of hemorrhagic enterotoxemia in a neonatal foal (7), was grown in medium CP-2V (13) for 6 h at 37°C to produce whole culture as a starting material. For alternate preparations the whole culture was centrifuged at 8000 x *g* for 20 min to obtain cells and cell-free supernatant fluid. The preparations, given intraduodenally through the cannula, were either the whole culture, cell-free supernatant fluid, or cells suspended in fresh CP-2V medium containing 20 g dextrin/300 mL medium. Cell counts were monitored by microscopy and the number of total cells per dose was adjusted to a uniform content of 7.5×10^{11}/300 mL.

Fresh soybean flour, ground from raw whole soybeans, was used as a trypsin inhibitor with 20 g added to the 300 mL inoculum. A further 20 g in 150 mL saline (0.15M NaCl) was given intraduodenally about 5 min prior to dosing with *C. perfringens* preparations. Control materials, including a *C. perfringens* type A strain, were also provided. These inocula are listed in the first column of Table I.

EXPERIMENTS

The inoculated lambs were observed continuously for clinical signs during the first day and four to five times daily for the next three days. Necropsy was performed on the dead animals and tissue samples and intestinal contents were collected for histological examination and toxicity tests, respectively. The intestinal fluids were clarified by centrifugation and filtration through a 0.22 m membrane and their serial (doubling) dilutions were used for toxicity titration in groups of mice (2-3 mice/dilution, 0.2 mL ea) by the intravenous route. Toxicity was expressed as minimum lethal dose (MLD/mL) when all inoculated mice died within 24 h. Diagnostic antisera (Burroughs Wellcome, Beckenham, Kent, U.K.), specific for *C. perfringens* types A, C and D, were used for toxin identification. Preinoculation serum samples from the lambs were tested for the presence of beta antitoxin by the toxin neutralization method in mice.

RESULTS

Table I summarizes overall clinical response of the experimental lambs to the inocula used. Six of the seven lambs which received either whole culture or cells in fresh medium (both inocula supplemented with soybean flour) died within two days; four having died on the day of inoculation. The first clinical sign noticed was general discomfort, including listlessness, shifting of body weight, loss of appetite and slight bloating. These signs appeared two to three hours postinoculation and persisted for approximately an hour. The animals then began to recover or deteriorate through lassitude and progressive depression, terminating in death without convulsions. The depressive state in those lambs which died 12 and 30 h postinoculation developed slowly during the terminal stages; the latter animal appeared almost normal by the end of the first day, except for anorexia. Five animals, including one *C. perfringens* type A control, exhibited transient discomfort and slight bloating, but recovered within a few hours. Ten doses which included whole culture, cell-free culture supernatant fluid, and medium-soybean control, did not produce any observable clinical response. Body temperature was not elevated in any animal.

Necropsy of the dead animals revealed severe hemorrhagic enteritis involving the small intestine posterior to the middle of the duodenum. The most pronounced change was in the ileum, decreasing in intensity in

TABLE I. Clinical Response of Lambs to Intraduodenal Administration of *Clostridium perfringens* Type C Preparations

	Inocula[a]	No. of Lambs	No. Affected[b]	No. Dead	Remarks
1	Whole culture	3	0	0	Remained normal
2	Whole culture + soybean	3	3	2	Discomfort in all at 2-3 h post-inoculation; two died at 7.5 and 30 h; one recovered
3	Cells + fresh medium	3	1	0	Discomfort in one at 3 h; two remained normal
4	Cells + fresh medium + soybean	4	4	4	Discomfort in all at 2-3 h; staggering and weakness in three shortly before death at 4.5-6 h; one died at 12 h
5	Cell-free culture supernatant	2	0	0	Remained normal
6	Cell-free culture supernatant + soybean	3	3	0	Discomfort in all at 2-3 h; two had unformed feces at 6-7 h
7	Cells of type A + fresh medium + soybean	2	1	0	Discomfort at 3 h; soft feces at 8 h; one remained normal
8	Fresh medium + soybean	2	0	0	Remained normal

[a]Dose: Whole culture or supernatant: 300 mL
Total cells: 7.5×10^{11}
Medium: 300 mL CP-2V + 20 g dextrin
Soybean flour: 20 g + predose of 20 g in 150 mL saline

[b]Obvious clinical signs or change of behavior

craniad direction in the small intestine. The contents were liquid, reddish brown and, in the acute cases, contained gelatinous clumps and a pseudomembrane. Less hemorrhage and more necrotic tissue was found in the intestine of the lamb that died 30 h postinoculation. Some gas was present in the intestinal tract, but extensive distention was not evident. The peritoneal cavity of all dead animals contained some straw-colored or blood-tinged fluid. A few animals had mild hydropericardium. Other organs appeared grossly normal. Animals which showed no signs or recovered were not killed for pathological examination.

Histological sections of the small intestine revealed extensive necrosis of the mucosa. The submucosa contained scattered pockets of emphysema. In some areas the detached pseudomembrane contained a layer of densely-packed bacterial cells resembling clostridial rods (Fig. 1). In lamb no. 12 which died 30 h postinoculation the pseudomembrane was absent, but the necrosis extended deeper into the submucosa than was seen in the acute cases. Liver, lungs and kidneys did not reveal any significant histological changes.

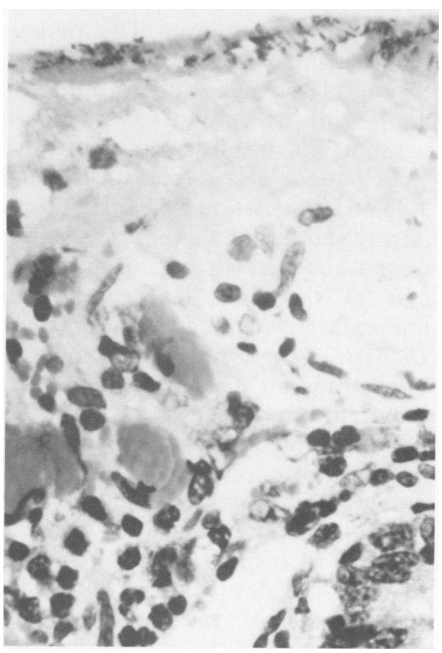

Fig. 1. Section of intestinal wall from middle of jejunum of lamb no. 135. Destruction of mucosa, formation of gas pockets in the necrotic mass, hemorrhage and a layer of bacterial cells are evident. H & E. X640.

TABLE II. Toxicity of Postmortem Intestinal Fluids from Lambs Intoxicated with *Clostridium perfringens* Type C

Sheep No.	Inoculum No. (Table I)	Toxicity of Inoculum MLD/mL	Death Post-inoculation h	Toxicity of Intestinal Fluids MLD/mL	
				Jejunum	Ileum
12	2	500	30	< 5	125
18	2	500	7.5	1000	1000
21	4	< 5	6	5000	4000
25	4	< 5	4.5	100	1000
135	4	< 5	12	200	500
191	4	< 5	5	500	2000

Table II shows the results of toxicity tests on the intestinal fluids collected at necropsy. Toxicity of all the fluids was completely neutralized by *C. perfringens* type C, but not by types A or D, antisera. None of the preinoculation sera neutralized type C toxin.

DISCUSSION

Without the use of soybean flour as a protease (trypsin and chymotrypsin) inhibitor neither the live culture nor the cell-free toxic culture supernatant fluid had any lethal effect on the animals in this experiment. These results suggest a role for normal digestive enzymes in protection against *C. perfringens* type C enterotoxemia and are generally similar to those of Lawrence and Cooke (11) who successfully produced necrotic/hemorrhagic enteritis in guinea pigs by inoculation of *C. perfringens* type C and administration of various dietary protease inhibitors.

Some differences in clinical response were evident between the two lethal inocula used in the present experiment. The response to cells in fresh medium was more uniform and more acute than it was to administration of whole culture. Toxicity tests (Table II) indicated that the cells given with fresh medium had multiplied in the gut and had produced large amounts of lethal beta toxin. The toxicity of whole culture inoculum in lamb no. 18 also increased, but by only twofold. This increase in toxicity could indicate additional growth and toxinogenesis in the intestine, or it could have been due to possible concentration of toxin by intestinal absorption of fluids. The relatively low toxicity of the intestinal fluid from lamb no. 12 may have been due to destruction or passage of the original toxin in the intestine over the long 30 h period.

Excluding possible species differences, the general response pattern in the present experiment resembled those obtained from experimental work on piglets. Field and Goodwin (9) were able to produce this disease by oral administration of *C. perfringens* type C whole culture, cells and toxin, but rarely if ever by washed cells. Similarly, inoculation of ligated intestinal loops of piglets with whole culture of the organism produced positive response whereas bacteria-free toxin or washed cells had no effect (1). In the present experiment on lambs the response suggested that enteric toxinogenesis was more significant than intoxication with preformed *C. perfringens* beta toxin.

The lesions in the small intestine of these lambs were of an acute, hemorrhagic nature rather than of necrotic character. This corresponds to natural cases of hemorrhagic enterotoxemia as seen in neonatal lambs, calves and foals (3-7). In field cases in lambs, Griner (5) reported the finding of hemorrhagic lesions in parts of the large intestine in addition to those in the small intestine. In piglets, however, several forms have been recognized, ranging from a peracute, hemorrhagic type in the youngest animals involved, to subacute and chronic forms characterized by predominantly necrotic lesions in animals a week or more old (1,14). This variation in tissue damage and resultant mortality rate is assumed to be related to factors such as the extent of bacterial growth and the rate of beta toxin production and its destruction

or neutralization in the intestine. If pancreatic secretion and protease production were not at full capacity in a brief postnatal period, inadequate levels of trypsin and/or chymotrypsin would make a newborn animal susceptible to the destructive effects of beta toxin, which is otherwise very sensitive to trypsin (15).

Another possible factor that has been suggested to contribute to the pathogenesis of type C enterotoxemia, at least in the case of piglets, is the presence of trypsin inhibitors in the colostrum (10). These may have an effect similar to the use of soybean flour in the present study.

Histological sections of the affected intestine in the present study revealed a concentration of clostridium-like rods on the necrotic mucosa. These were presumably *C. perfringens* type C organisms. Arbuckle (16) demonstrated experimentally in piglets that an attachment of these organisms to villous epithelium occurs as a first step in the pathogenesis of type C enterotoxemia. Similar bacterial attachment or abundant proliferation has been seen in natural cases of necrotic or hemorrhagic enterotoxemia of piglets (1,2), lambs (5), calves (3), foals (6,7) and in man (17). This suggests that it may not be by the presence of beta toxin alone that this organism produces disease, but that active involvement of multiplying clostridia on the villous epithelium may be important. This view is supported by the results of the present experiment. It is not known whether attachment by these cells would still occur in the presence of trypsin or colostrum-transferred antibeta antibodies; these substances do not necessarily inhibit the growth of *C. perfringens*.

The evidence presented here indicates also that whole soybean flour is a suitable product for use in experimental models studying pathogenesis and immunity in *C. perfringens* type C enterotoxemia.

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