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Clostridium perfringens Type A Infection of Ligated Intestinal Loops in Lambs

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Clostridium perfringens type A suspended in fresh medium was injected into ligated intestinal loops of lambs. Within 7 hr after inoculation, the fluid volume of the loops increased up to seven times. No significant accumulation of fluid occurred in loops receiving grown culture, culture supernatant fluid, or medium alone. α-Antitoxin injected along with *C. perfringens* in fresh medium into intestinal loops did not prevent the accumulation of fluid. It is concluded that α-toxin plays no major role in *C. perfringens* type A enteritis.

Clostridium perfringens type A may cause enteritis in man. The illness usually occurs a few hours after ingestion of food contaminated with large numbers of *C. perfringens* cells. The main symptoms are diarrhea and abdominal pain (10). The disease was reproduced experimentally in lambs when *C. perfringens* cells were introduced orally or intraduodenally into the animals (7). Strains of *C. perfringens* type A have also been suspected of causing natural outbreaks of enteritis in lambs (7, 12, 17).

The factors responsible for the disease are still unknown. Nygren (14, 15) reported that the intestinal passage time in mice and monkeys was significantly reduced after feeding culture filtrates of *C. perfringens* or *Bacillus cereus* or after administration of phosphoryl choline; they also reported that the contractions of isolated rabbit intestines could be increased by adding the same preparations to the organ bath. Nygren concluded (15) that *C. perfringens* food poisoning was mediated by the activity of α-toxin, a lecithinase C, through formation of phosphoryl choline. The hypothesis was plausible for the following reasons. (i) α-Toxin is the only lethal toxin of *C. perfringens* type A, and lethal toxins are involved in all other known diseases that are caused by various types of *C. perfringens*. (ii) *B. cereus* causes a food-poisoning disease in man similar to that associated with *C. perfringens* (6) and also produces a lecithinase C (18). (iii) Choline and choline esters are known to stimulate the peristaltic activity of intestines (4). However, some doubt was cast upon Nygren's results and hypothesis by the following findings. (i) Filtrates from *C. perfringens* cultures caused no discomfort in human volunteers, whereas cells from the same cultures produced the typical food-poisoning syndrome (2). (ii) Weiss et al. (20) found no effect of phosphoryl choline on the intestinal passage time in mice and monkeys. (iii) *C. perfringens*, strain S-79 produced only traces of α-toxin in vitro, but effectively caused diarrhea in humans (8) and lambs (7). Several other strains of *C. perfringens* implicated in food-poisoning outbreaks were also found to produce little or no demonstrable α-toxin in liquid culture (9, 11). (iv) Immunization of lambs with the complete anaculture of a food-poisoning strain of *C. perfringens* resulted in three- to fourfold increases in the α-antitoxin levels of the blood, but afforded no protection against subsequent intraduodenal challenge with cells of the same strain.

In the present studies, which were undertaken to prove or to disprove the involvement of α-toxin in the enteric disease, α-antitoxin and *C. perfringens* cells were introduced into lamb intestines, and the effect of the antitoxin on the course of the disease was determined. Since the experimental design described previously (7) would require large quantities of antitoxin, an attempt was made to produce local enteritis by infecting ligated intestinal loops of lambs with *C. perfringens*. The "intestinal loop technique" has been used to study the enteropathogenicity of *Vibrio cholerae* and *Escherichia coli* in rabbits and piglets (1, 3, 13).

MATERIALS AND METHODS

Experimental animals. Lambs of the Rambouillet breed, 8 months old and weighing approximately 30 kg, were used. Prior to the experiments, the animals

were confined in corrals and barns and fed a maintenance diet of good quality alfalfa hay and free choice of cobalt-iodine-salt mixture. The animals were free from gastrointestinal parasites.

Intraduodenal administration of bacterial cells. C. perfringens type A strains S-79 and 80535 (8) were grown at 37 C for 16 hr. The medium (CP-2V) contained 2% each of dextrin, proteose peptone, and yeast extract, 0.1% cysteine·HCl, 0.2% Na_2HPO_4, and 0.1% K_2HPO_4. The *p*H was adjusted to 7.2. Subcultures in CP-2V with 7% inoculum were grown for 4.5 hr. A 30-ml amount of culture was mixed with 220 ml of deaerated medium CP-2V, supplemented with an additional 3% dextrin, 2% proteose peptone, and 0.4% glucose (CP-2VS), and was introduced into lambs via a duodenal tube (7). The number of cells introduced per animal was approximately 1.2×10^{10} for strain S-79 and 4.5×10^{10} for strain 80535. To avoid bubbling and excessive aeration of the liquid, the intravenous drip apparatus (7) was modified by connecting the flutter valve with the free air space in the inverted flask via plastic tubing. Lambs were killed in the early stages of enteritis, i.e., about 10 hr later. Samples of the intestinal contents were taken along the small intestines and stored immediately at −15 C for subsequent assay of α-toxin.

Ligated intestinal loop technique. The animals were starved for 3 days, except for a little drinking water that was given on the second day. Under local procaine infiltration anaesthesia, and with a midline approach, laparotomy was carried out aseptically. From a point about 1 meter from the cecum and proceeding anteriorly, ligated segments 15 to 20 cm long were prepared in the ileum and jejunum by use of a synthetic monofilament suture material. The ligatures were placed so that the blood supply from the mesenteric vessels to each loop remained intact. The inocula were injected into alternate loops; the test loops were thus separated by uninjected interloops. The abdomen was then closed, and the animal was killed and examined 7 or 16 hr later.

Unless otherwise stated, the ligated loops were inoculated with mixtures of 0.25 ml of *C. perfringens* culture and 2.25 ml of medium CP-2VS. When the effect of α-antitoxin was investigated, the loops received 0.25 ml of culture, 2.0 ml of CP-2VS, and 0.25 ml of trivalent therapeutic gas gangrene antitoxin (Parke, Davis and Co. Detroit, Mich.). The mixtures contained 10^8 cells of strain S-79 or 3.5×10^8 cells of strain 80535, and 175 international units of *C. perfringens* α-antitoxin per loop. In control loops, the bacterial culture was replaced with medium CP-2V, and the solution of antitoxin was replaced with 0.85% NaCl. For examination, samples of the loop contents were removed and stored immediately at −15 C. In the 7-hr experiments, 16-hr cultures were used; in the 16-hr experiments, 4.5-hr subcultures were used.

Preparation and administration of α-toxin. C. perfringens type A strain PB-6K (supplied by L. DS. Smith, Montana State College, Bozeman) was grown in medium CP-2V at 37 C for 4 hr with the *p*H controlled automatically at 7.0 (16). The culture was centrifuged at 5 C and $12,000 \times g$ for 10 min. The supernatant fluid contained 400 mouse MLD of α-toxin per ml. Samples of this fluid were stored immediately at −15 C. A volume of 500 ml was concentrated to 31 ml by dialysis against polyethylene glycol 20,000 (Fisher Scientific Co., Pittsburgh, Pa.) at 5 C without measurable loss of lecithinase activity. The preparation was stored at −15 C. The volumes injected per intestinal loop were 2.5 ml (1,000 MLD) for the original supernatant fluid, and 1.25 ml (8,000 MLD) for the concentrated preparation. Three loops in each of five lambs received 1,000 MLD, and three loops in each of two lambs received 8,000 MLD of α-toxin. The control loops received equivalent volumes of medium CP-2V only.

Assay of α-toxin and antitoxin. α-Toxin was measured by injecting 0.5 ml of serially diluted samples intravenously into 22-g white mice, and by the lecithovitellin (LV) test of van Heyningen (19), modified as follows: 0.2 M tris(hydroxymethyl)-aminomethane(Tris)buffer was used as diluent; the samples were incubated at 37 C for 25 min, and the reaction was stopped with 5 ml of 1% ethylenediaminetetraacetate (*p*H 7.0).

α-Antitoxin was assayed as described previously (7) with the following minor variations: the supernatant fluid of strain BP-6K was used as a source of α-toxin, and was diluted 1:40 to contain 40 MLD/ml; 0.2 M Tris buffer was used as a diluent for all preparations. Prior to the assays, the insoluble ingredients of the intestinal contents were removed by centrifugation at 5 C and $8,000 \times g$ for 10 min. The liquid contents were diluted 1:200 and 1:400 prior to the addition of standard α-toxin. These high dilutions were necessary because α-toxin was also inactivated when mixed with intestinal control samples at dilutions up to 1:100.

Incubation of α-antitoxin with C. perfringens culture and sheep-intestinal contents. Mixtures of 1.4 ml of deaerated medium CP-2V, 0.2 ml of a 16-hr culture of strain S-79, and 0.4 ml of gas gangrene antitoxin were incubated at 37 C for 5 hr. The control samples contained 0.85% NaCl instead of antitoxin. Optical densities were measured at 600 mμ in a Spectronic-20 colorimeter (Fisher Scientific Co.) after 2, 4, and 5 hr of growth.

Mixtures of 0.8 ml of soluble intestinal contents from normal lambs and 0.2 ml of the antitoxin were incubated at 37 C for 4 hr.

Phosphoryl choline administration. Nine intestinal loops were prepared in each of three lambs. Three loops each per lamb received 3.6 mg (15 μmoles) or 36 mg (150 μmoles) of calcium phosphoryl choline (Nutritional Biochemicals Corp., Cleveland, Ohio) in 2.5 ml of medium CP-2V, or they received 2.5 ml of CP-2V only. The volumes of the loop contents were measured 7 hr later.

Each of two lambs received intraduodenally 200 or 300 mg of calcium phosphoryl choline in 250 ml of CP-2V. The controls received 250 ml of CP-2V only.

RESULTS

α-Toxin in intestines. Two lambs were infected intraduodenally with strains S-79 and two with

strain 80535. The lambs were killed about 10 hr later. Three of the four lambs had profuse diarrhea, and the fourth produced normal feces. The intestinal contents were assayed for α-toxin by the LV test and by mouse injections. No toxin could be detected.

Incubation of α-antitoxin with C. perfringens culture and sheep intestinal contents. The preparation of gas gangrene antitoxin had no effect on the growth of *C. perfringens* strain S-79. Conversely, no reduction in the α-antitoxin content was found as a result of incubation with growing cultures of strain S-79, or with sheep-intestinal contents.

Infection of intestinal loops with C. perfringens. Figure 1 shows a series of intestinal loops distended with fluid 16 hr after injection of *C. perfringens* strain S-79 mixed with medium CP-2VS. No significant accumulation of liquid occurred in the interloops or in the loops receiving medium alone. Distended loops were also obtained with *C. perfringens* strain 80535. None of the distended loops showed gross signs of acute or hemorrhagic inflammation, and no alpha toxin was detected in their fluids.

Positive intestinal loops were not produced in all lambs. Quantitative differences in the response to *C. perfringens* were also found between equally treated intestinal loops in the same lamb, depending on the location of the loops along the small intestine. The relative position of the infected loops showing the largest increase varied between individual lambs.

To determine which of the fractions in the mixture injected were responsible for the observed volume increases, the preparations listed in Table 1 were injected into the intestinal loops in each of two lambs. The series of inoculations was repeated once in each lamb. The intestinal loops were examined 16 hr later. Of the loops that had received

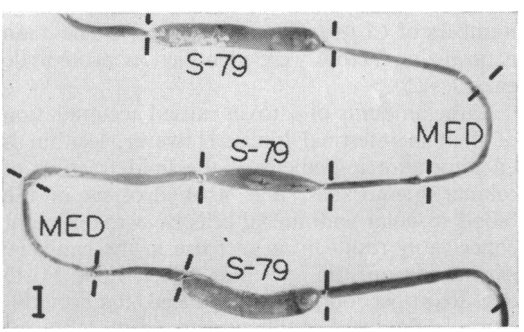

FIG. 1. *Accumulation of fluid in ligated intestinal loops infected with C. perfringens type A strain S-79. The labeled loops received 2.25 ml of medium CP-2VS plus 0.25 ml of culture (S-79) or 0.25 ml of CP-2V (MED). Incubation period, 16 hr.*

TABLE 1. *Conditions for accumulation of fluid in intestinal loops*[a]

Material injected (2 ml)	Accumulation of fluid
(a) Whole culture	−
(b) Culture supernatant fluid	−
(c) Medium CP-2V	−
(d) Whole culture (0.25 ml) + CP-2VS (1.75 ml)	+
(e) Heated[b] culture (0.25 ml) + CP-2VS (1.75 ml)	−

[a] Incubation period, 16 hr; *C. perfringens* strain, S-79.

[b] At 100 C for 2 min.

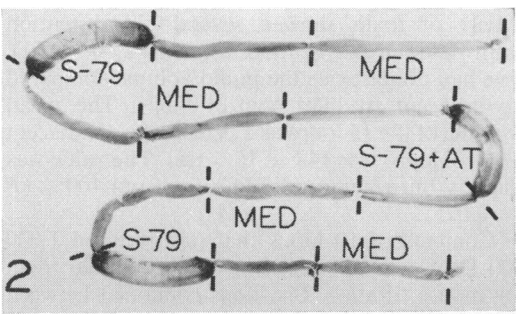

FIG. 2. *Effect of gas gangrene antitoxin on the accumulation of fluid in ligated intestinal loops infected with C. perfringens type A strain S-79. The labeled loops received 2.0 ml of medium CP-2VS plus 0.25 ml each of the following preparations: C. perfringens culture + 0.85% NaCl (S-79), culture + antitoxin (S-79 + AT), or CP-2V + 0.85% NaCl (MED). Incubation period, 7 hr.*

preparations a, b, c, and e (Table 1), none showed any significant increase in liquid content. The loops receiving preparation d were consistently distended with fluid, indicating that the loop distention is caused by a mixture of *C. perfringens* culture and fresh medium.

When the intestinal loops were examined 16 hr after inoculation, considerable amounts of gas, as well as fluid, had accumulated inside. To exclude any physical effect of gas on the accumulation of liquid, all subsequent experiments were terminated 7 hr after inoculation.

Effect of α-antitoxin on accumulation of fluid in intestinal loops. Figures 2 and 3 offer a comparison of intestinal loops that received culture S-79 in fresh medium CP-2VS with and without α-antitoxin. No significant differences were apparent between the two sets of positive loops. Volume measurements also revealed no significant differences. The infected loops shown in Fig. 3 were moderately distended and contained very little

gas. The accumulation of liquid in the loops is therefore due to factors other than physical dilatation of the intestinal wall. The increases in liquid content of the infected loops were of the order of 700% (Fig. 2) and 300% (Fig. 3). No significant increase in liquid volume was found in the loops that received medium and saline only (Fig. 2).

Samples of intestinal loop contents were assayed for residual α-antitoxin by the LV test. Between 40 and 85% of the original antitoxin was recovered after the 7-hr incubation period.

Effect of α-toxin. The possible role of α-toxin was investigated further by injecting this toxin into intestinal loops and measuring the volumes of the contents 7 hr later. Loops that had received 8,000 MLD of toxin showed several-fold distention with fluid. Of the 15 loops receiving 1,000 MLD, five had about twice the liquid volume compared to adjacent medium control loops. The mean volume of the 15 loops as a percentage of adjacent control loops was 142 $\pm$ 16% (SE). The value was slightly but significantly different from 100% (P = 0.025).

Contents of 10 loops that had received 1,000 MLD of α-toxin were assayed for residual toxin by mouse titration. Six loops contained between 2 and 12% of the toxin injected, the remaining four loops had no measurable toxin. The lethal toxin was completely neutralized with *C. perfringens* type A antiserum. No correlation was apparent between the volumes of liquid contents and the residual α-toxin of individual loops.

Effect of phosphoryl choline. Phosphoryl choline injected into intestinal loops was without measurable effect. Lambs receiving 200 mg of calcium phosphoryl choline intraduodenally continued to produce normal stools; the lambs receiving 300 mg of calcium phosphoryl choline produced soft feces for a few hours, but did not develop diarrhea

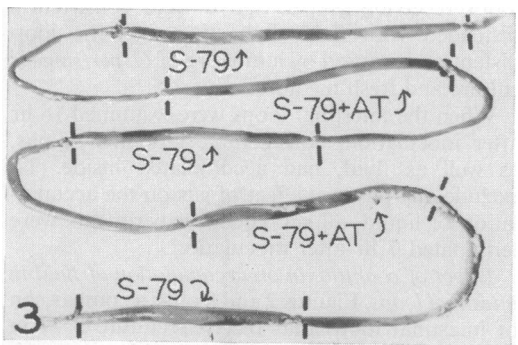

FIG. 3. *Moderately distended intestinal loops inoculated with C. perfringens type A strain S-79 with (S-79 + AT) or without (S-79) gas gangrene antitoxin. Inoculations as in Fig. 2, except that MED loops were omitted. Incubation period, 7 hr.*

DISCUSSION

Fluid accumulated rapidly in ligated intestinal loops inoculated with a mixture of *C. perfringens* type A culture and fresh medium, but no accumulation of fluid occurred in loops receiving whole cultures, culture supernatant fluids, or medium only. The same results were obtained in producing experimental enteritis in lambs with nonligated intestines (7; *unpublished data*). It appears, therefore, that the same factors are involved in the response of the individual ligated loops to injection with *C. perfringens*, and in enteritis caused by *C. perfringens* in whole intestines.

Of the α-antitoxin injected along with *C. perfringens* into intestinal loops, over 40% of the original activity and between 3 and 6 international units per ml of loop fluid could be recovered from the positive loops after the experiment. This finding indicates that any α-toxin produced by *C. perfringens* during the 7-hr incubation period would be readily neutralized by the antitoxin. Since the antitoxin itself had no effect on the accumulation of fluid in infected loops, the α-toxin can no longer be regarded as a major factor in *C. perfringens* enteritis. This conclusion is substantiated by the absence of α-toxin in the small intestine of lambs killed at the early stage of diarrhea caused by *C. perfringens* type A.

The absence of measurable amounts of α-toxin from infected loops and from the intestines of diseased lambs may be explained by the rapid inactivation of toxin by the intestinal contents in the absence of antitoxin, and by the production of relatively small amounts of α-toxin by strains S-79 and 80535 in liquid culture (8, 9).

Goudie (5) was also unable to demonstrate α-toxin in human diarrheal fluids containing large numbers of *C. perfringens* type A cells. The main neutralizing factors were identified as proteolytic enzymes (5).

Large amounts of α-toxin caused accumulation of fluid in intestinal loops. However, lecithin is an important ingredient of the lipid fraction of cellular membranes, and its hydrolysis in the blood-vascular endothelial cells by α-toxin might conceivably result in an increase in the capillary permeability of the intestines. When 1,000 MLD of α-toxin per loop was introduced, the accumulation of fluid was small compared with loops infected with *C. perfringens*. On the other hand, 6 of 10 loops receiving α-toxin had measurable amounts of residual toxin 7 hr later, whereas no toxin could be demonstrated in loops infected with *C. perfringens*. Consequently, despite the

demonstrated effect of α-toxin on the sheep intestines, these experiments provide no indication that α-toxin plays a significant role in *C. perfringens* enteritis. They are, rather, in agreement with our conclusion that the toxin has no major role in the disease.

Nygren (15) reported that as little as 30 μmoles of phosphoryl choline per kg of body weight decreased the passage time in mice and caused diarrhea in a monkey. In our experiments, up to 150 μmoles of phosphoryl choline per loop caused no accumulation of fluid. Considering that the lambs weighed about 30 kg, and that the individual loops covered less than 5% of the small intestinal tract, the relative amount of phosphoryl choline introduced per loop was well above that used by Nygren (15). The maximal amount of phosphoryl choline introduced per loop would be derived from about 100 mg of lecithin upon hydrolysis. It is doubtful that so much substrate would be available in an intestinal loop.

Relatively large amounts of phosphoryl choline (1,250 μmoles) introduced intraduodenally into lambs produced soft stools for a few hours. This effect is slight compared with the effect of *C. perfringens*. Our experiments, therefore, give no indication that phosphoryl choline may be involved in *C. perfringens* type A enteritis.

Since this manuscript was submitted for publication, Duncan et al. (J. Bacteriol. **95**:1560, 1968) reported accumulation of fluid in ligated intestinal loops of rabbits in response to infection with *C. perfringens*.

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