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Mechanism of Action of the Enteropathogenic Factor of *Clostridium perfringens* Type A

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Cell extract of an enteropathogenic strain of *Clostridium perfringens* type A was administered intravenously to lambs, rabbits, and guinea pigs. Lambs developed transitory diarrhea, lacrimation, salivation, nasal discharge, lassitude, and dyspnea in 1 to 5 hr after inoculation. Large doses of the inoculum caused rapid onset of the clinical signs and subsequent death. Examination of dead animals revealed intensely hyperemic small intestinal mucosa and some congestion in the liver, lungs, spleen, and kidneys. Rabbits showed excessive salivation, frequent defecation, tranquility, and dyspnea, followed by death. Guinea pigs became weak and died in 15 min to 7 hr. Congestion was evident in lungs, liver, spleen, and in the small intestine. In lambs and guinea pigs tested, atropine and epinephrine alleviated the clinical signs. Intradermally injected cell extract caused an immediate increase in capillary permeability and subsequent erythematous reaction without necrosis in the skin of guinea pigs. It is hypothesized that in the enteric infection *C. perfringens* enteropathogenic factor acts on the small intestine causing increased capillary permeability, vasodilation, and increased intestinal motility.

Certain strains of *Clostridium perfringens* type A can cause enteritis in man and animals (9). The disease has been reproduced experimentally in lambs (9, 11) and rabbits (5), and enteric reactions have been demonstrated in ligated intestinal loops of these animals (7, 10). A characteristic finding in the experimental disease is transitory diarrhea in an intact animal and accumulation of clear, straw-colored, or blood-tinged fluid in ligated intestinal loops. The enteric response has been found to be due to an enteropathogenic factor associated with growth and sporulation of *C. perfringens* cells under suitable conditions (6, 11, 12). In my investigations, the experimental disease in lambs neither conferred immunity against subsequent attacks nor was it preventable in parenterally immunized animals which possessed neutralizing serum antibody against the enteropathogenic factor (*unpublished data*). In the experimental disease in lambs, bacterial invasion of the intestinal mucosa does not occur (9), and the clinical response can be reproduced by cell-free extracts of the causative organism (11).

This work was conducted to study the systemic and clinical effects of the enteropathogenic factor of a *C. perfringens* cell extract in lambs, rabbits, and guinea pigs and to relate these observations to the probable action of this factor.

MATERIALS AND METHODS

Inocula. *C. perfringens* type A, strain NCTC 8239, used in an earlier study (11), was grown in the sporulation medium (DS medium) of Duncan and Strong (4) for 3 hr at 37 C. One part of this culture was then added to nine parts of fresh warm DS medium in 4-liter flasks and incubated for 7 hr at 37 C. During this time, about 70% of the cells sporulated as determined by phase-contrast microscopy. The cells were harvested, washed, and sonically treated, and an extract was prepared, as previously described (11). The extract was sterilized by filtration through a 0.45-μm membrane filter and was designated "enteropathogenic cell extract" (ECE). This ECE contained 420 mg of total protein/100 ml (biuret method), and it was stored at -20 C.

Control cell extracts were similarly prepared from three "classical" strains of *C. perfringens* type A which did not produce the enteropathogenic effect (11, 12) in lambs. Another control extract was made from strain NCTC 8239 grown in asporogenic medium CP-2V (10). Pharmaceutical preparations used were epinephrine (1:1,000 and 1:10,000 solutions) and atropine sulfate (50 mg/100 ml of 0.85% NaCl).

Experimental animals and inoculation. Seven-month-old Suffolk lambs weighing 30 to 35 kg and 6-month-old Cheviot lambs weighing 20 to 25 kg were used in these experiments. New Zealand White rabbits and guinea pigs weighing 2.2 to 2.7 kg and 350 to 360 g, respectively, were also used.

Portions of the frozen ECE were thawed and diluted

to 10% concentration in 0.85% NaCl and injected intravenously (iv). The dose administered was expressed as the volume of the undiluted ECE per kilogram of body weight. Undiluted ECE was used only for intradermal (id) inoculations in guinea pigs. The control extracts were injected in undiluted form. Epinephrine and atropine sulfate were administered subcutaneously (sc) to the lambs; guinea pigs were given these drugs either sc or iv. The maximal volume of total iv inoculum per guinea pig did not exceed 0.8 ml.

Inoculated animals were observed continuously for 8 to 11 hr and were checked periodically during the next 2 days. Those animals that died were subjected to necropsy within 20 min after death. Tissues were fixed in buffered 10% Formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

The effect of ECE on capillary permeability was tested in 10 guinea pigs. Undiluted ECE was injected id in volumes of 0.05 ml per site into depilated albino guinea pigs at various time intervals ranging from 0.5 to 24 hr. Then 0.5 ml of 1% Evans blue (British Drug Houses Ltd., Poole, England) in 0.85% NaCl was introduced by the iv route; final id injections were made immediately thereafter. The formation of skin lesions was observed for up to 48 hr. The skin reactions were also examined histologically.

Tissue changes in the intestinal mucosa of dead animals resulting from iv treatment were compared histologically with those of "positive" ligated intestinal loops of lambs. For this purpose, ligated intestinal loops (10) were injected with 0.05 to 0.5 ml of ECE and allowed to react for 6.5 hr. Specimens from four lambs, comprising a total of 25 loops with various degrees of reaction, were collected and examined histologically.

RESULTS

Systemic reaction in lambs. The clinical response to iv injections of the *C. perfringens* ECE is shown in Table 1. The intensity of the clinical signs corresponded to the dose. At dose levels of 0.006 to 0.010 ml/kg, the only observable clinical abnormality was soft unformed feces, arbitrarily classified as +. These were passed once or twice only at 5 to 8 hr after inoculation. At larger dosages, the feces became voluminous and watery (+++). Animals receiving 0.030 to 0.032 ml of ECE per kg developed diarrhea 1.5 to 3 hr after inoculation. This lasted for about 2 hr; thereafter, no feces were passed for 10 to 20 hr.

A number of animals exhibited signs indicative of abdominal discomfort prior to the onset of diarrhea: short periods of struggling, kicking at abdomen, and grinding of teeth. These signs of discomfort alternated with quiescent periods of 3- to 6-min duration which gradually lengthened, became more pronounced, and developed into general lassitude. In such cases, an animal appeared as if deeply tranquilized, but still having full muscular coordination. Dyspnea either coincided with the onset of discomfort and lassitude or began shortly afterward. It varied in form from hyperpnea to expiratory grunt; the latter degree appeared in animals receiving larger doses and in all of those which later died.

Other clinical effects were lacrimation, mucous nasal discharge, and occasional salivation. These signs lasted for 1 to 2 hr in survivors. Periodic shivers were also observed in severely affected animals. All animals refused to eat. Rectal temperature in affected animals was 102.5 to 103.5 F (38.6 to 39.8 C), which was considered to be within the normal range.

Three lambs inoculated with 0.030 ml of ECE per kg (not shown in Table 1) were injected sc with 0.25 ml of a 1:1,000 solution of epinephrine about 30 min after inoculation. Clinical signs in the epinephrine-treated animals appeared to be milder and of shorter duration as compared with those of the untreated ones. The intensity of diarrhea was judged ++ in one and + in the other two animals. After a 4-day rest, the inoculations were repeated, but two of the lambs were treated with 1.5 mg of atropine sulfate sc 30 min postinoculation. The two treated animals developed signs similar to, but milder than, those seen in lambs injected with epinephrine. The untreated lamb had ++ diarrhea within 4 hr and also showed all of the other clinical signs previously observed. Clinical signs were not observed in any of the animals injected with control cell extracts in volumes of up to 0.3 ml/kg.

The three lambs that died (none of these had diarrhea) revealed on necropsy an extremely hyperemic mucosa of the jejunum and ileum, including the ileo-cecal valve. Slight mucosal bleeding was evident in the ileum. Less intense hyperemia was seen in the duodenum and cecum, and to a minor degree in the anterior portion of the large intestine. Two animals that received the largest dose of ECE revealed ascites and hydrothorax; the fluid volumes were approximately 150 and 50 ml, respectively. All carcasses showed various degrees of pulmonary, hepatic, and splenic congestion. Mesenteric lymph nodes were slightly edematous.

Histological examination of the jejunum and ileum revealed pronounced hyperemia and partial loss of the mucosal surface epithelium. The blood vessels and capillaries in the lamina propria were engorged with erythrocytes (Fig. 1 and 2). A few blood vessels in the denuded surface were noted to be ruptured, but no hemorrhages were found in the stroma. About 40% of the surface epithelium seen in sections had sloughed off. The lungs, liver, and spleen were congested. In the liver, congestion was localized around the portal triads, and the kidneys revealed hyperemic

TABLE 1. *Clinical signs in animals after intravenous administration of C. perfringens strain 8239 cell extract (ECE)*

Animal	Dosage (ml/kg)	No. of animals tested	No. of animals responding (and time of first appearance of signs)[a]							
			Diarrhea	Lacrimation	Salivation	Nasal discharge	Discomfort	Lassitude	Dyspnea or hyperpnea	Death
Sheep	0.006–0.010	3	2 (5–8), +[b]	0	0	0	0	0	0	0
	0.012–0.016	2	1 (3.5), ++	2 (2)	0	2 (2–2.5)	2 (1–2)	2 (1–2.5)	2 (1–3)	0
	0.020–0.025	6	4 (3–12), +–++	4 (1–4)	1 (3)	5 (1–4.5)	5 (2–4)	2 (1.5–3)	4 (1.5–5)	1 (11)
	0.030–0.032	3	3 (1.5–3), +++	3 (1.5–2)	1 (2)	2 (1.5–2)	2 (1)	3 (2)	3 (1.5–2.5)	0
	0.050–0.10	2	0	2 (0.4–1)	2 (0.4–1)	2 (0.4–1.5)	0	2 (0.3–0.5)	2 (0.4–1)	2 (0.6–2.5)
Rabbits	0.020–0.031	3	0	0	3 (2–4)	0	0	3 (2–3)	3 (0.5–2)	0
	0.038–0.042	3	2 (1–1.5), +	0	3 (0.6)	0	0	3 (0.5–1)	3 (0.5–0.8)	3 (2.5–10)
Guinea pigs	0.043	4	0	0	0	0	0	2 (1–2)	0	0
	0.055	4	0	0	0	0	0	4 (1–1.5)	0	1 (7)
	0.070	3	0	0	0	0	1 (0.5)	3 (0.5–0.8)	2 (1–4)	2 (3–5)
	0.085	5	0	1 (0.5)	0	0	1 (0.5)	5 (0.1–0.2)	2 (0.3)	5 (0.4–0.7)
	0.110	5	0	1 (0.6)	0	0	0	5 (0.1–0.2)	3 (0.2–0.4)	5 (0.3–0.7)

[a] Minimum and maximum time in hours; minutes shown as approximate decimals of an hour.
[b] Relative intensity of diarrhea from + to +++, given as minimum and maximum if a range existed.

cortical rays. Other organs, including lymph nodes, salivary glands, and the brain, did not show significant histological changes.

Systemic reaction in rabbits and guinea pigs. The clinical response in rabbits differed from that in sheep (Table 1). All of the rabbits tested developed marked salivation which lasted 1 to 2 hr. The animals defecated frequently, and unformed feces were passed by two of the three rabbits receiving the higher dosage of ECE. Tranquility, lassitude, and hyperpnea were exhibited by all animals. Lacrimation, nasal discharge, and abdominal discomfort, which were prominent signs in lambs, were not evident in rabbits and guinea pigs. In the dead animals, pulmonary, hepatic, and intestinal congestion was present but was less intense than in the

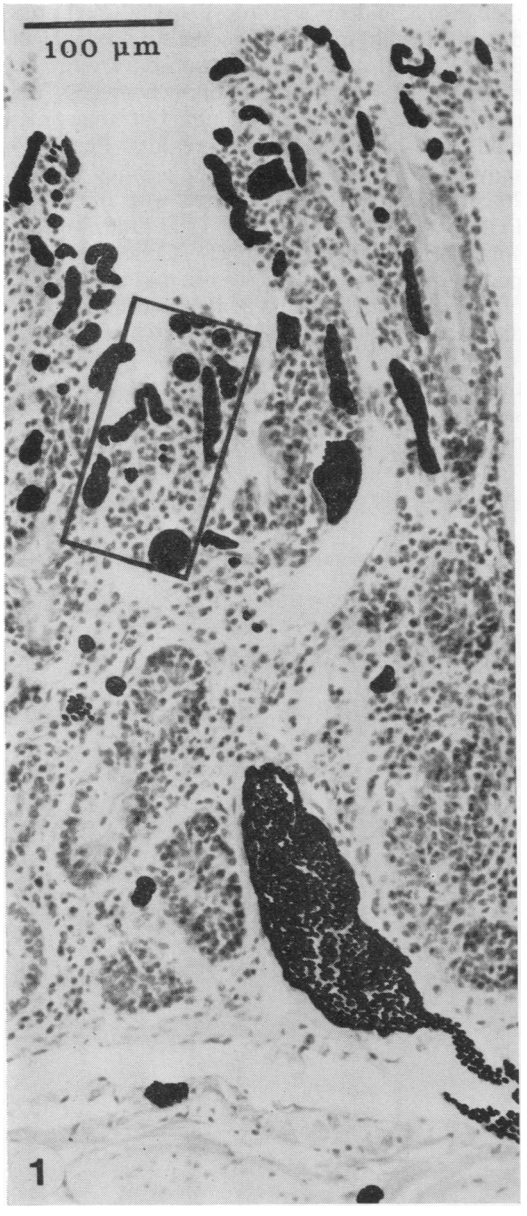

FIG. 1. *Photomicrograph of the jejunum of a lamb inoculated intravenously with C. perfringens enteropathogenic cell extract, showing hyperemic lamina propria devoid of surface epithelium. The blood-filled vessels are shown retouched black. The lined rectangle area is shown at a higher magnification in Fig. 2.*

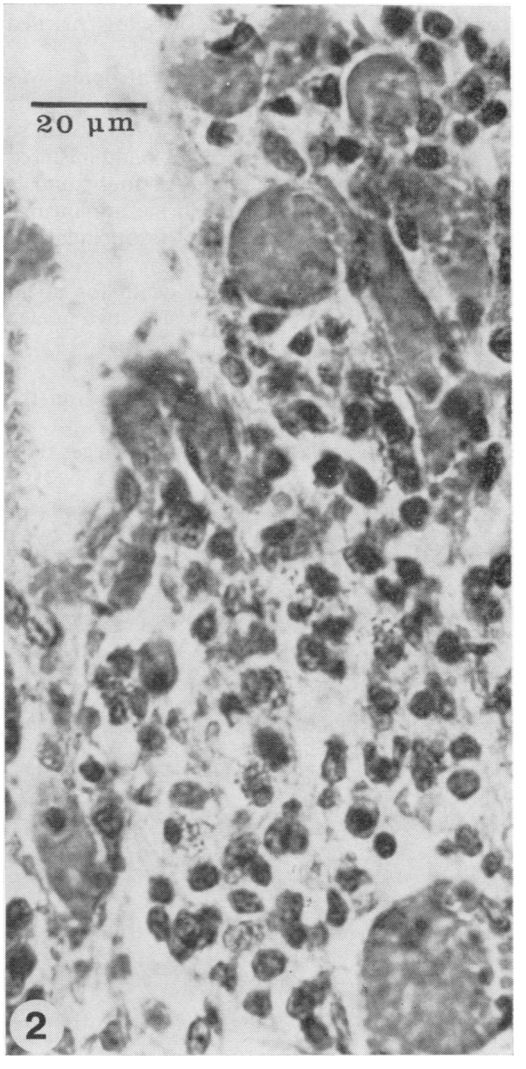

FIG. 2. *Portion of Fig. 1 at higher magnification; not retouched.*

sheep. The intestinal mucosa was intact. Control cell extracts and the effect of epinephrine and atropine were not tested in rabbits.

Guinea pigs exhibited lassitude and died, but the effective dose was proportionately higher on the body-weight basis than the effective dose for sheep and rabbits (Table 1). Other clinical signs in guinea pigs were either absent or very mild. Control preparations were uniformly ineffective. Dead animals revealed congestion in the liver, spleen, lungs, mesentery, and small intestine. In some carcasses, the peritoneum was blood-tinged. Histological appearance of the congested tissues was essentially similar to that seen in sheep, although hyperemia in the lamina propria of the small intestine was less marked and the surface epithelium was intact.

The effect of epinephrine and atropine was investigated in guinea pigs inoculated with 0.085 ml of ECE per kg. Either 0.2 ml iv or 0.3 ml sc of epinephrine (1:10,000) was administered about 10 min postinoculation. All four animals tested in each mode of epinephrine administration died between 1.5 and 2 hr postinoculation. Atropine sulfate administered iv in the amount of 0.04 mg per animal delayed death for up to 3 hr in four of the six animals tested; two guinea pigs survived.

Ligated intestinal loops in lambs. The degree of fluid accumulation in the ligated loops and the appearance of the fluid corresponded with the extent of mucosal damage. (Usually, larger dose of ECE caused greater fluid accumulation.) Dark, blood-tinged fluid contained numerous erythrocytes and epithelial cell casts. Loops distended with a large amount of clear fluid (20 to 60 ml/20 cm of loop length) contained fewer epithelial cells and no erythrocytes (Fig. 3). Loops distended with small and moderate amounts of fluid (5 to 20 ml/20 cm) contained very few or no epithelial cells. Sections of such mucosa did not reveal damaged epithelium, but sometimes moderate hyperemia was present. Figure 4 shows a section near the tip of a villus from a 20-cm loop, which was injected with 0.1 ml of ECE, distended with 18 ml of clear fluid, without apparent histological changes. Concurrent hyperemia was not constant; even some loops devoid of surface epithelium and containing erythrocytes did not reveal a blood-engorged vascular network at the time of examination (6.5 hr postinoculation). Ligated control loops injected with either saline or nontoxic cell preparations revealed neither loss of surface epithelium nor apparent hyperemia. No bacterial invasion of mucosa was detected in any of the ligated loops.

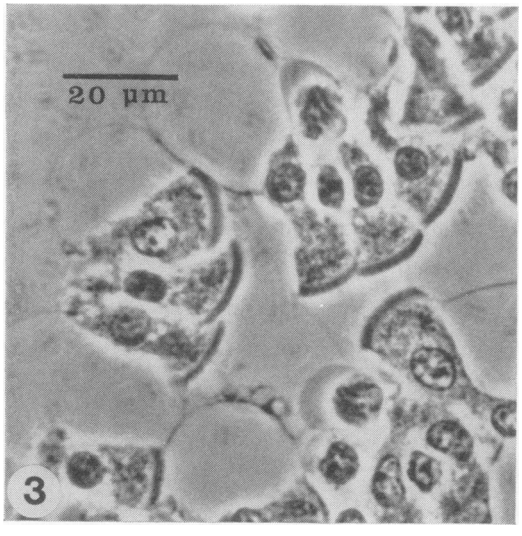

FIG. 3. *Phase-contrast photomicrograph of cellular sediment of accumulated fluid from a heavily distended ligated intestinal loop of a lamb. Unstained wet preparation.*

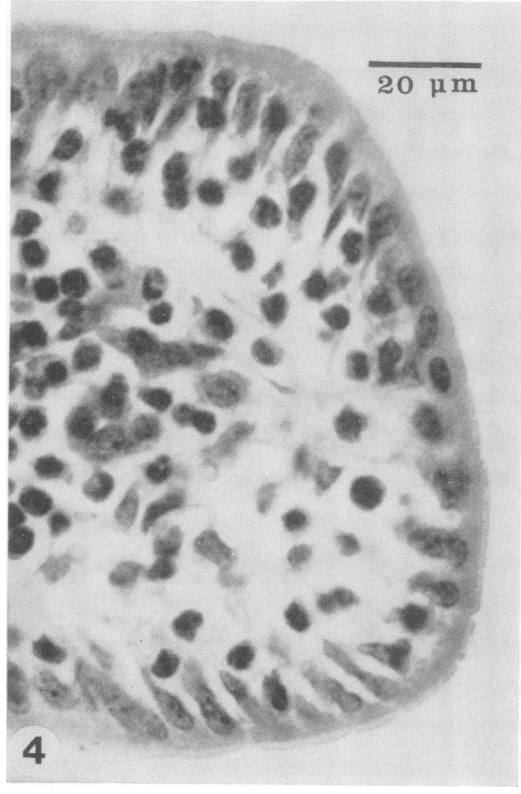

FIG. 4. *Tip of a villus from a moderately distended intestinal loop of a lamb.*

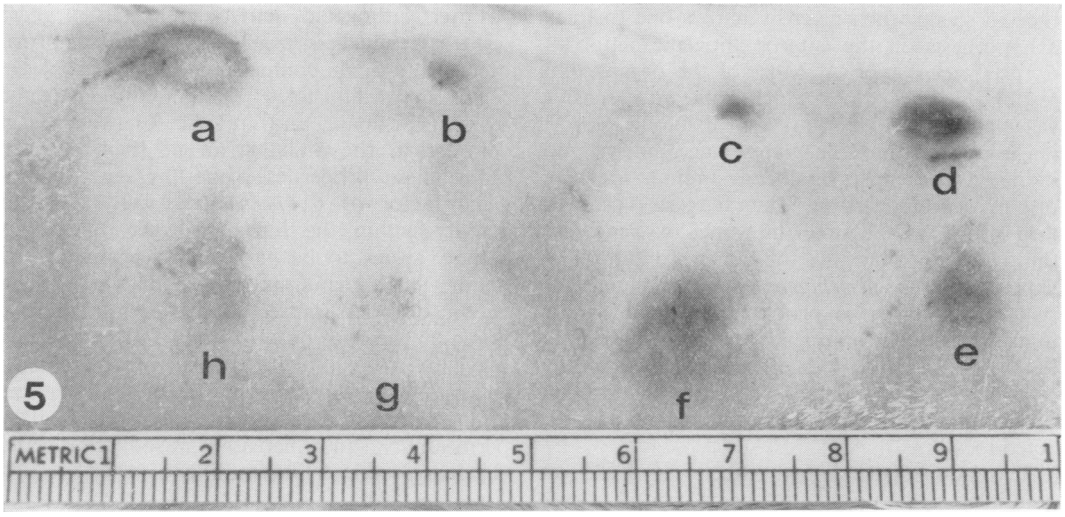

FIG. 5. *Intradermal reactions in a guinea pig to test capillary permeability. Photograph taken 20 min after iv administration of Evans blue. Preparations injected id at the following hours prior to Evans blue injection: (a) enteropathogenic cell extract (ECE), 0 hr, blue ring; (b) control cell extract of strain 8239 grown in asporogenic medium, 0 hr, small blue spot; (c) 0.85% saline, 0 hr, small blue spot; (d) ECE, 24 hr, red area, no blue; (e) ECE, 6 hr, red area, no blue; (f) ECE, 2 hr, red central area, slight bluish tinge surrounding it; (g) saline, 2 hr; (h) as in b, 2 hr.*

Intradermal reactions in guinea pigs. An area of erythema developed at the site of ECE injection. A very faint reddening could sometimes be detected 20 min after the id injection; most reactions became visible in 1.5 to 2 hr and measured 5 to 8 mm in diameter. The lesions became darker in color, but did not increase in size after about 4 hr. Maximal darkening of the reaction was sometimes attained in 24 hr; it appeared purplish-red and nonedematous. After slight induration, these reactions resolved themselves without abscessation or necrosis within the following 2 to 3 days.

Histological examination of the skin sections taken from lesions 1.5 to 48 hr old showed that the initial erythematous reactions were located in the dermis and were followed by mild infiltration by polymorphonuclear leukocytes in reticulum immediately above the cutaneous muscle layer (in about 4 hr). A few extravasated erythrocytes were noted, but no apparent hemorrhage was evident. Necrosis of the tissue was absent.

Guinea pigs injected with Evans blue developed ringlike blue zones around a clear central area within 15 to 20 min after subsequent id inoculation with ECE (Fig. 5). The blue rings rarely developed around the erythematous lesions 2 hr old and were not seen in lesions older than 8 hr. Lesions 0.5 hr old developed rings similar to the immediately reacting injections; 1-hr lesions appeared fainter and diffuse rather than ringlike. Control sites injected with saline and nontoxic cell extracts sometimes formed a small nonspreading blue spot at the needle puncture immediately after inoculation, indicating trauma. These spots did not occur in control areas injected 2 or more hr previously (Fig. 5).

DISCUSSION

The results of this study indicate that the enteropathogenic factor of *C. perfringens* type A can exert a significant effect on the intestine and cause diarrhea when introduced into the blood stream. This should not mean, however, that absorption of the factor from the intestinal lumen into the blood stream is necessary before diarrhea can occur. The intravenous approach seemed to locate responsive tissues or target organs by exposing them via systemic circulation to the suspected toxic preparation. Although the extract tested was a crude preparation, the possibility that substances other than the enteropathogenic factor contributed significantly to the observed responses appears remote. Control extracts from vegetative cells that lacked the enteropathogenic activity in ligated intestinal loops were also inactive systemically. Also, there was no cause to suspect the presence of the classical exotoxins of *C. perfringens* in the ECE. The ability of small doses of ECE to cause diar-

rhea in sheep when given iv allows one to form a hypothesis on the enteropathogenic action of *C. perfringens* type A. Most of the organs that responded were those which have an extensive vascular bed or which are rich in blood supply, i.e., the small intestine, lungs, liver, spleen, and kidneys. The most significant finding in these organs was congestion. This suggests vasodilation which, in the small intestine, may increase the passage of fluid into the lumen. In natural or experimental *C. perfringens* enteritis, the enteropathogenic factor is probably confined to the intestine only. In animals with ligated intestinal loops, the reaction was always confined to the test loops and no obvious changes occurred in other visceral organs, indicating that the factor acted locally.

The additional clinical effects of ECE on glandular secretory activity (salivation, lacrimation, nasal discharge) suggest parasympathomimetic properties. Agents that possess such properties are also known to increase intestinal motility, intestinal secretion, and vasodilation (13). In some lambs and rabbits which did not develop marked diarrhea, the frequent defecation and larger than normal amount of passed feces could be interpreted as an apparent increase in intestinal motility.

Both epinephrine and atropine alleviated the clinical signs brought about by ECE, but atropine had a greater effect than epinephrine. The latter drug is a vasoconstrictor, whereas atropine is considered an antagonist to pharmacological activity of many parasympathomimetic agents (13). This symptomatic alleviating effect of these drugs may add further support for the parasympathomimetic mode of action of the enteropathogenic factor of *C. perfringens*.

Skin tests in guinea pigs showed that ECE caused an immediate brief increase in capillary permeability and subsequent erythema without fluid exudation. Also noteworthy was the absence of necrosis in the skin, even with relatively large doses of ECE. This is in contrast to an activity of the alpha toxin of *C. perfringens*, which is strongly dermonecrotic. The latter toxin also produces hemorrhage and increases capillary permeability with subnecrotizing doses (8). The ability of ECE to increase skin capillary permeability may also aid in the passage of fluid into the intestinal lumen in the diarrheal process. This type of action has been found with *Vibrio cholerae* enterotoxin (3), which also produces erythema and increases capillary permeability in the skin (1, 14); however, these manifestations are slower in developing, but longer lasting, 16 to 28 hr (2), as compared with the range of 15 to 120 min for the *C. perfringens* ECE studied.

Enteropathogenic activity of *C. perfringens* cell-free products is largely unaffected by normal ovine intestinal contents (*unpublished data*), relatively resistant to *p*H changes, and unaffected by trypsin, lipase, and amylase (6), which is in contrast to the alpha toxin of this organism (15). These properties make the enteropathogenic factor of *C. perfringens* well suited to activity within the intestinal tract. Since this activity seems to be parasympathomimetic, the major local manifestation is diarrhea. Such enteric disorders caused by this factor either in domestic animals or in human food poisoning may, therefore, be viewed as a form of enterotoxemia with the following suggested changes taking place in the intestine: (i) increased capillary permeability, (ii) increased vasodilation, and (iii) increased intestinal motility.

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