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Fluid Accumulation in Mouse Ligated Intestine Inoculated with *Clostridium perfringens* Enterotoxin

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Clostridium perfringens enterotoxin, when inoculated into the ligated intestinal loop of mice, caused marked distension due to fluid accumulation. The increase in weight of the intestinal loop was proportional to the log dose of enterotoxin within a range from 1 to 16 μg. The fluid accumulation was arrested by washing the loop with saline or by injection of the specific anti-enterotoxin serum into the loop 5 or even 30 min after inoculation of the enterotoxin. A significant increase in weight of the loop was found as early as 10 min after inoculation of the toxin. These results may suggest that enterotoxin is neither bound firmly to the mucosal membrane nor permeates into the cells of the intestinal wall. The mouse intestinal loop test is economical, simple to perform, and applicable for quantitative determination of the enteropathogenic activity of *C. perfringens* enterotoxin.

Certain strains of *Clostridium perfringens* cause food poisoning in humans. Several methods have been developed for purifying *C. perfringens* enterotoxin from extracts of sporulating cultures of type A (5, 6, 16, 20) and C (18) strains, and its biological activity has been characterized. The enterotoxin is the primary agent of diarrhea in *C. perfringens* food poisoning (4). The enterotoxin produced by sporulating cells acts on the mucous membrane of the intestinal tract and alters the water transportation mechanism across the intestinal wall (10). Little is known, however, about the exact site of action of enterotoxin and the mechanism involved in diarrhea.

Several methods are available to detect the biological activity; however, it seems important to develop a more sensitive and quantitative assay method to study the enteropathogenicity of the enterotoxin. The purposes of the present studies were to characterize the response of the ligated intestines of mice to *C. perfringens* enterotoxin and to demonstrate its usefulness as a convenient technique for further studies.

MATERIALS AND METHODS

Purification of enterotoxin. *C. perfringens* type A enterotoxin was purified from the extract of sonically disrupted sporulating cells of strain NCTC 8798 grown for 8 h in Duncan-Strong medium (1) by the method described previously (16). The purified enterotoxin gave a single band in polyacrylamide gel electrophoresis and also a single precipitation line in agar double diffusion against rabbit antiserum prepared with it. The toxin, dialyzed against saline, was diluted appropriately with saline, dispensed into Wassermann tubes each with 0.5 to 2 ml, and kept frozen until used.

Protein determination. Protein determination was performed by the method of Lowry et al. (9) with crystalline bovine serum albumin (Armour Pharmaceutical Co., Phoenix, Ariz.) as the standard.

Antiserum. Anti-enterotoxin serum was obtained by subcutaneous injections of 10-μg doses into rabbits, the primary injection with Freund complete adjuvant and the subsequent ones without adjuvant. The second injection was given 5 weeks after the first one; subsequent injections were given at 3-week intervals over a period of 6 months. Blood was taken 4 days after the second and subsequent injections. The crude antiserum was used for the in vitro neutralization test given in Fig. 6. The immunoglobulin G fraction, obtained by gel filtration of the crude antiserum on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), was used for the in vivo neutralization test given in Fig. 8. The anti-enterotoxin serum was titrated by passive hemagglutination (22), and the titer is expressed by the reciprocal of the highest dilution causing hemagglutination.

Intestinal loop test. Female white mice (strain ddYS) weighing 20 to 25 g were starved for 24 to 28 h, during which time water only was provided. Before the operation, the mice were injected intraperitoneally with 2 mg of sodium pentobarbital (Abbott Laboratories, North Chicago, Ill.) per 20 g and fastened on a board. An incision was made along the abdominal midline. The small intestines were withdrawn and ligated at a distance of about 6 cm from the pylorus with a piece of cotton thread. Several loops were made downward with interspaces of about 0.5-cm long. Immediately after ligation, each loop was injected with 0.1 ml of enterotoxin or saline (for control) with a syringe attached with a hypodermic needle. The abdomen was closed by a stitch, and the animal was left as such on the board. After an appropriate period, the animal was killed by cutting the thoracic aorta under

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anesthesia, and the abdomen was reopened. The individual loops were excised and put in a petri dish containing filter paper dipped in saline. Each loop was hung on a fixed clip and stretched by putting another clip weighing 2 g on the other end of the loop. Then, each loop was measured in length and weight. The weight/length ratio (milligrams/centimeter) or the net increase in weight of the loop (milligrams) was used to express the intensity of the reaction.

RESULTS

Fluid accumulation in the inoculated loop. The intestinal loops inoculated with enterotoxin were filled with clear fluid and markedly distended after a certain period. A false positive reaction rarely occurred for 4 h after injection of saline. Positive fluid accumulation was easily recognized; however, weighing the intestinal loop gave more objective results.

Effect of length of the loop on fluid accumulation. It was difficult to measure such a small volume of the secreted fluid in the mouse intestinal loop. The response of the intestinal loop to the enterotoxin was expressed, at first, in a weight/length ratio (milligrams/centimeter). The relationship between the length of the loop and the weight/length ratio is shown in Fig. 1. The enterotoxin-injected loops gave values higher than 60 mg/ml, as compared with those injected with saline that gave values lower than 50 mg/ml. The values of the inoculated loop were dependent on the length of the loop; short loops gave higher values, and longer ones gave lower values. The variance could be minimized by making loops of the same length, but it was impracticable to do so in such a small animal like the mouse. As seen in Fig. 1, the weight/length ratio seemed to be inversely proportional to the length of the loop. Therefore, the quantity of fluid accumulated in a loop (F) may be calculated by the following formula: $F = W_t - (W_c/L_c) \times L_t$ (milligrams), where W_t is the weight of the loop inoculated with enterotoxin (milligrams), W_c is the weight of the control loop (milligrams), L_t is the length of the loop inoculated with enterotoxin (centimeters), L_c is the length of the control loop (centimeters), W_c/L_c denotes weight in milligrams/length in centimeters of the control loop, and $(W_c/L_c) \times L_t$ indicates the weight of the loop before the injection of enterotoxin. Thus, F is the weight of the fluid accumulated in the loop after the inoculation of enterotoxin.

As shown in Fig. 2, most F values were within a certain range (190 to 340 mg and 240 to 400 mg when 10 μg and 20 μg of enterotoxin were injected, respectively) when the lengths of loops were between 2.5 and 9 cm. However, shorter loops tended to give smaller F values than this range, and longer ones gave irregular values upon inoculation of 20 μg of enterotoxin (Fig. 2B). The values were fairly constant when the lengths of loops were between 2.5 and 9 cm (Fig. 2A and B). Thus, calculations by the above formula gave least variance to quantify the fluid accumulated in the loops.

Fluid accumulation in different regions of the small intestine. Figure 3 shows the responses to 10 μg of enterotoxin of loops made in various regions of the small intestine. Loops made at distances between 6 and 18 cm from the

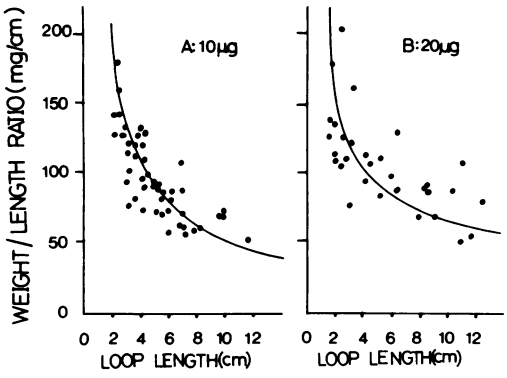

FIG. 1. *Weight in milligrams/centimeter in response to the length of the loop after injection of enterotoxin. Each loop was injected with 10 μg (A) or 20 μg (B) of enterotoxin. They were measured in weight and length in 90 min.*

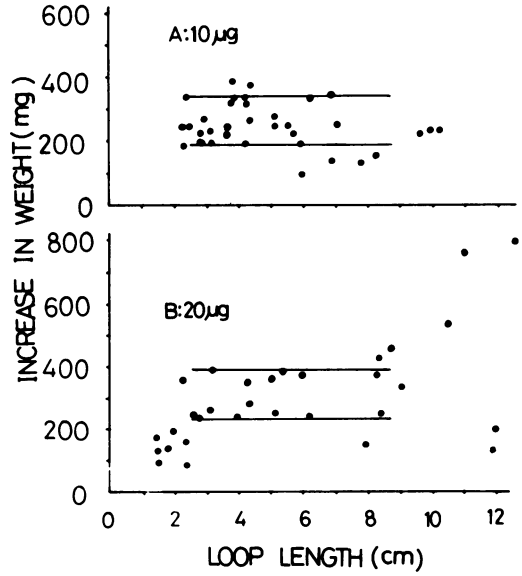

FIG. 2. *Net weight of the fluid accumulated due to the injection of enterotoxin into loops of various lengths. The calculations were made by the formula mentioned in the text.*

pylorus gained and retained more weight than those made in lower regions of the intestine. The weight/length ratios of control loops injected with saline differed from 35 to 50 mg/cm, depending upon the individual animal, but it was nearly constant in loops in the same animal if loops were made in the region between 6 and 18 cm from the pylorus. Therefore, three of four loops made in the region of the intestine between 6 and 18 cm from the pylorus were injected with enterotoxin of the same dose, and the fourth was injected with saline. The mean of the values obtained with the three loops was used to express the enterotoxic activity in subsequent experiments.

Time course of fluid accumulation. The time course of fluid accumulation in response to 10 μg of enterotoxin is shown in Fig. 4. The fluid accumulation was maximum in 60 min after inoculation of enterotoxin, but variation in the values was the least in 90 min. The period of 90 min seemed to be the most suitable for determination of fluid accumulation and was used in the following experiments.

Dose response. The increase in weight of the inoculated loop was almost proportional in response to log dose of enterotoxin ranging from 1 to 16 μg as shown in Fig. 5. The total fluid accumulated in three loops was 900 mg per mouse as a result of injection of 16 μg or more of enterotoxin into each loop, which may be the maximum amount of the body fluid that can be secreted.

Antiserum prepared against *C. perfringens* enterotoxin neutralized the enteropathogenic activity of the toxin (Fig. 6). The inhibitory activity was dependent on the concentration of the antiserum used. Normal rabbit serum did not decrease the response to enterotoxin.

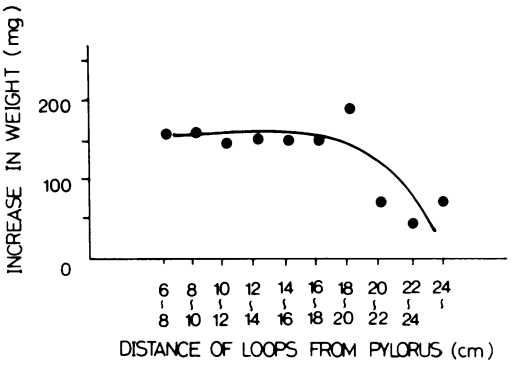

FIG. 3. *Effect of enterotoxin injection (10 μg) on the fluid accumulation in the loops made in the different regions of the intestine. Each point represents the mean of five to seven loops measured in 90 min. Each distance was measured from the center of the loops.*

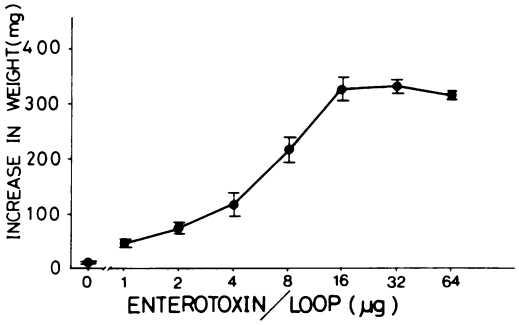

FIG. 5. *Net increase in weight of the loop in response to the various doses of enterotoxin measured in 90 min. Each point represents the mean of 15 loops. The vertical bar represents standard error.*

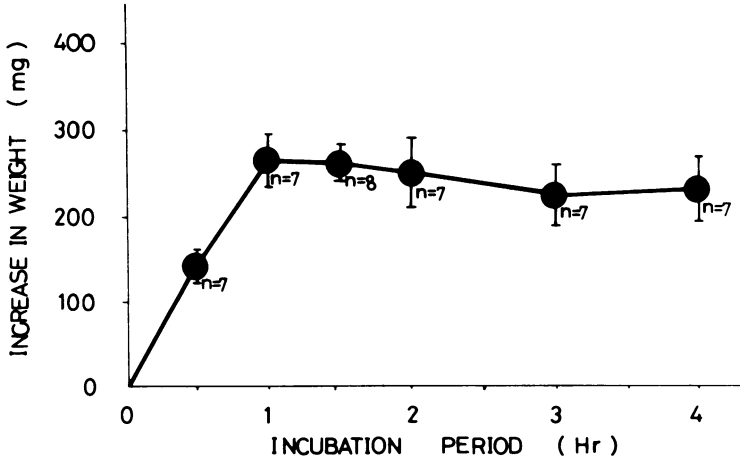

FIG. 4. *Time course of fluid accumulation in the mouse intestinal loop injected with 10 μg of enterotoxin. Each point represents the mean ± standard error of 21 to 24 loops.*

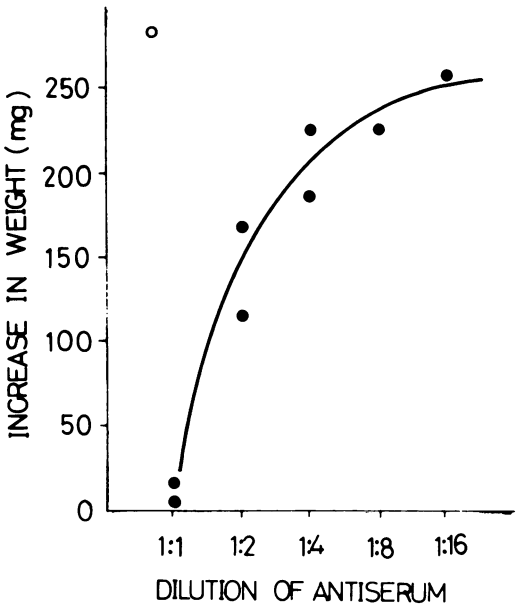

FIG. 6. *In vitro neutralization of enterotoxin with antiserum. Enterotoxin (200 μg/ml) was mixed with an equal volume of serially diluted anti-enterotoxin (●) with a passive hemagglutination titer of 64,000 or normal rabbit serum (○). The mixture was incubated for 30 min at 35°C, and a 0.1-ml portion was injected into a loop. Each point represents the mean of three loops in one mouse.*

Effect of washing the lumen and of injection of antibody into the loop after injection of enterotoxin. To study the mode of action of *C. perfringens* enterotoxin, attempts were made to remove the inoculated enterotoxin by washing the intestinal lumen. A ligated loop was inoculated with 0.1 ml containing 16 μg of enterotoxin. The luminal fluid was taken out, and the lumen was washed with 0.6 ml of saline with a syringe. To the washed loop, saline was added to the equal volume of the fluid that had been withdrawn. The fluid accumulation was markedly arrested by washing 5 or even 30 min after inoculation of enterotoxin (Fig. 7).

The concentrated specific antibody (0.1 ml of the immunoglobulin G fraction) that had a potency of neutralizing 20 μg of enterotoxin was injected into the ligated loop 5 or 30 min after injection of 8 μg of enterotoxin. The antitoxin given even 30 min after the enterotoxin inoculation stopped fluid accumulation. The already accumulated fluid at the time of antitoxin administration was absorbed back (Fig. 8). The immunoglobulin G fraction of normal rabbit serum did not have such an effect.

Rapidity of fluid accumulation in the loop. To determine whether the fluid accumulation is the primary action of enterotoxin, the lag time was measured by injecting 10 μl containing 20 μg of enterotoxin. As shown in Fig. 9, a significant increase in weight of the loop was found as early as 10 min after inoculation.

DISCUSSION

Mild diarrhea was noticed by feeding mice with sterile filtrate of *C. perfringens* culture by some workers (13, 17), whereas others (23) failed to confirm these findings. Hobbs et al. (8) also failed to induce symptoms in mice by administering *C. perfringens* culture directly into the stomach. It has not been confirmed whether *C. perfringens* grows and produces enterotoxin in the mouse intestine. On account of these inconsistent results, little attention has been paid to the mouse as an experimental animal for studying the enteropathogenicity of *C. perfringens* or its enterotoxin.

The present results demonstrated that *C. perfringens* enterotoxin induced distinct fluid accumulation in the ligated intestinal loops of mice. According to Torres-Anjel et al. (21), suckling mice developed watery diarrhea after intragastric inoculation of 0.5 μg or less enterotoxin.

We found that the mouse intestinal loop responded very rapidly to enterotoxin. Fluid accumulation appeared to start right after injec-

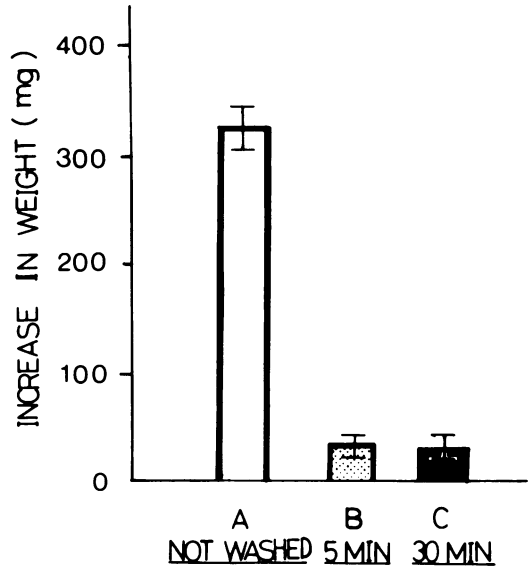

FIG. 7. *Effect of washing on fluid accumulation in the mouse intestinal loop after injection of 16 μg of enterotoxin. Already accumulated fluid was withdrawn from the loop, if there was any, and washed with 0.6 ml of saline with a syringe, 5 (B) min or 30 (C) min after inoculation. Each value is the mean ± standard error of nine loops.*

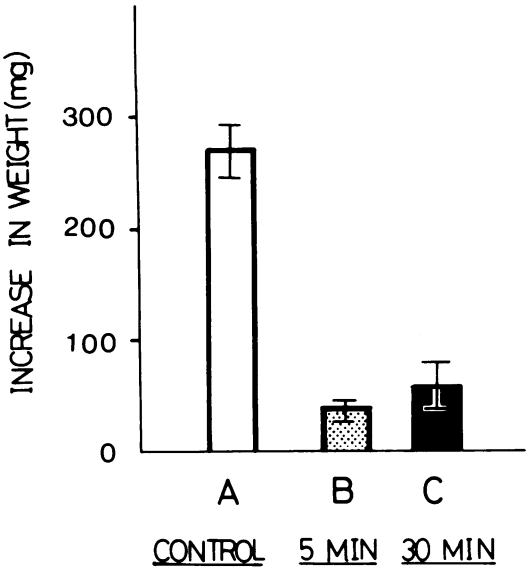

FIG. 8. *Effect of anti-enterotoxin immunoglobulin G (0.1 ml) with a passive hemagglutination titer of 128,000 on fluid accumulation administered after 5 or 30 min of injection of 0.05 ml containing 8 μg of enterotoxin. For control (A), a normal rabbit IgG fraction (0.1 ml) was administered 5 min after injection of the same quantity of enterotoxin. Each value is the mean ± standard error of nine loops.*

tion of enterotoxin because a measurable quantity of fluid was accumulated within 10 min. Intestinal loops inoculated with 10 μg of enterotoxin showed maximum fluid accumulation in 90 min, which is in conformative to Hauschild et al. (7), who stated that the rabbit intestinal loop tests should be read after 90 min rather than after 6 h. Such a rapid response to enterotoxin has also been found in other biological systems, such as the increased skin permeability within 10 to 20 min (12, 19) and mouse lethality within 30 min (16), whereas the response to cholera enterotoxin occurred after a lag period ranging from 30 min to more than 90 min (2). It is well known that such a long lag period is due to indirect reaction after the stimulation of adenylate cyclase which results in the enhancement of secretion of water.

Washing the lumen of the intestinal loop even after exposure to enterotoxin for a considerable period ceased fluid accumulation. The specific anti-enterotoxin serum also inhibited fluid accumulation in the same way. On the other hand, only 5-min exposure to cholera enterotoxin of perfused rabbit intestinal loop is sufficient for maximum water accumulation (J. T. Goodgame, J. G. Banwell, and T. R. Hendrix, Fed. Proc. **31**: 259, 1972). Once the loop is exposed to cholera enterotoxin, specific antibody is unable to check the water accumulation (11), which is due to the fact that cholera enterotoxin is bound specifically to the epithelial cells of the intestine (14). The present results show that *C. perfringens* enterotoxin does not bind firmly to the epithelial cells of the intestine but only alters the transportation mechanism of electrolytes and water. The results also indicate that continuous exposure of the lumen to *C. perfringens* enterotoxin seems necessary for fluid accumulation and its persistence in the intestine. The present findings may explain the transient symptoms of *C. perfringens* food poisoning in humans.

Several methods have been reported for the determination of biological activity of *C. perfringens* enterotoxin. The mouse intravenous injection and skin tests are easy to perform and highly sensitive. Yet, the intestinal loop test is valuable to determine the enteropathogenic activity. Rabbit, lamb, calf, and chicken intestines have been shown to be susceptible to *C. perfringens* cells or enterotoxin. About 6 to 125 μg of enterotoxin is required to induce fluid accumulation in these experimental animals, whereas the intestinal loop test in mice can detect *C. perfringens* enterotoxin quantitatively and in such a small quantity as 1 μg.

The use of mouse intestinal loops as test tools

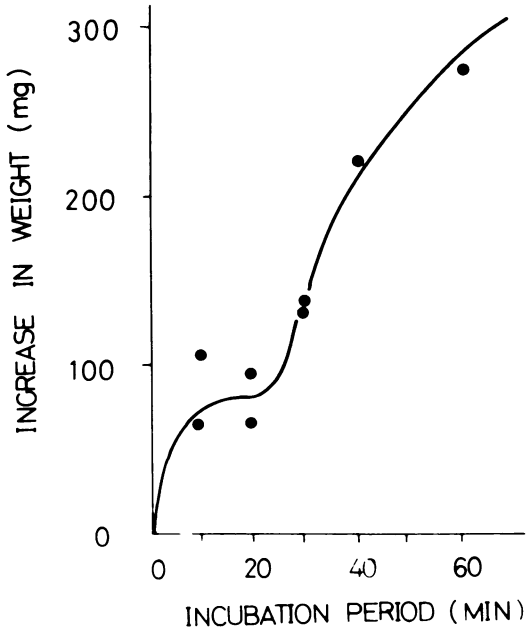

FIG. 9. *Time course (first 60 min) of fluid accumulation in the mouse intestinal loop. Each loop was injected with 20 μg of enterotoxin in 10 μl. Each point represents the mean of three loops.*

for *C. perfringens*, *Escherichia coli* (15), *Vibrio cholerae* (3), and other enterotoxin-producing bacteria should be encouraged because this test can detect enterotoxin in minute quantities and mice are very economical and easy to rear and handle.

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