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Pathogenesis of Salmonellosis: Salmonella Exotoxins

Annual Progress Report

(9/1/78-9/1/79)

Johnny W. Peterson, Ph.D.

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University of Texas Medical Branch
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This annual report summarizes research progress accomplished during the second year of the above mentioned research contract. The bulk of this report consists of two recently prepared manuscripts, which summarize most of the more significant and successful observations made

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during this project period. The manuscripts describe successful efforts to quantitate the cholera toxin-like toxin present in crude culture filtrates of several Salmonella species. Addition of Mitomycin C to cultures was observed to increase the amount of the heat labile, cholera toxin-like toxin as detected by the Chinese hamster ovary cell (CHO) assay. Since the toxin appearing in culture filtrates in the presence of Mitomycin C was heat labile, and was neutralized by addition by monospecific cholera antitoxin or gangliosides, the toxin was considered to be the same as that causing an increase in vascular permeability as reported previously. Using these techniques, attempts were made to perform surveys of selected isolates of Salmonella for production of the toxin. Two Salmonella isolates were selected to study the effect of selected culture media, temperature, oxygen tension, and trypsin on release of toxin.

Other efforts during this project period have been directed toward determining whether the heat labile Salmonella toxin has a direct, enterotoxic effect on the small intestine of experimental animals. The second manuscript describes the initial report that partially purified preparations of the toxin cause accumulation of fluid in ligated intestinal loops of adult rabbits. This response is blocked by heating the toxin preparation (100°C for 10 minutes) or by preincubation with monospecific cholera antitoxin. In addition, we have substantiated an earlier report that immunization of rabbits with procholeragenoid will protect them against fluid loss from intestinal loop challenge with Salmonella typhimurium. The latter observation tends to support the concept that a heat labile, cholera toxin-like enterotoxin could be involved in the loss of fluid and electrolytes from the intestine during salmonellosis.

Several other lines of investigation are in progress and are discussed in the latter part of this report. Some of the data appear clear cut but are not

ready for publication, while other results have been obtained that cannot be readily interpreted at this time. Despite occasional frustrations involved in this work, we feel that each week a better understanding of the characteristics and role of Salmonella enterotoxin is obtained.

Several serotypes of Salmonella were shown to release increased amounts of a cholera toxin-like toxin during culture in vitro with Mitomycin C. Filter sterilized culture supernatants containing the toxin caused elongation of Chinese hamster ovary cells, which could be blocked by heating the supernatants at 100°C for 15 minutes, or adding mixed gangliosides or monospecific cholera antitoxin. When Mitomycin C was not added to the Salmonella cultures, little or no detectable toxin was released. Optimal production of toxin was observed in the presence of 0.5 g/ml of Mitomycin in shake flask cultures of CYE medium, Syncase, or peptone saline at 37°C. Meat infusion media (HI and BHI) resulted in poor toxin yield. Culture filtrates frequently could be diluted 1:8 and still result in elongation of CHO cells, but were too dilute to cause fluid accumulation in ligated intestinal loops of adult rabbits without further concentration.

A partially purified preparation of the delayed skin permeability factor from Salmonella typhimurium exhibited enterotoxic activity in ligated intestinal loops of adult rabbits. The fluid accumulation response was blocked by heating the toxin preparation at 100°C for 10 minutes or by addition of monospecific cholera antitoxin. In addition, rabbits immunized with procholeragenoid were protected against fluid loss following live cell challenge with live Salmonella typhimurium. Antisera to cholera toxin or Salmonella toxin neutralized cholera toxin as well as Salmonella toxin.

Progress Report
DAM D17 77 C 7054

Annual Progress Report

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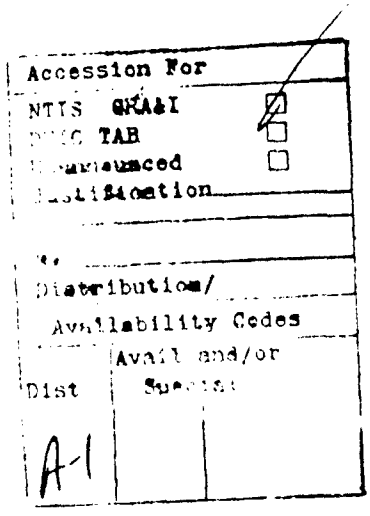

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I. Summary

This annual report summarizes research progress accomplished during the second year of the above mentioned research contract. The bulk of this report consists of two recently prepared manuscripts, which summarize most of the more significant and successful observations made during this project period. The manuscripts describe successful efforts to quantitate the cholera toxin-like toxin present in crude culture filtrates of several Salmonella species. Addition of Mitomycin C to cultures was observed to increase the amount of the heat labile, cholera toxin-like toxin as detected by the Chinese hamster ovary cell (CHO) assay. Since the toxin appearing in culture filtrates in the presence of Mitomycin C was heat labile, and was neutralized by addition of monospecific cholera antitoxin or gangliosides, the toxin was considered to be the same as that causing an increase in vascular permeability as reported previously. Using these techniques, attempts were made to perform surveys of selected isolates of Salmonella for production of the toxin. Two Salmonella isolates were selected to study the effect of selected culture media, temperature, oxygen tension, and trypsin on release of toxin.

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II. A.

A Cholera Toxin-Like Enterotoxin Released by *Salmonella* Species in the Presence of Mitomycin C

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Running Head: *Salmonella* Enterotoxin

Acknowledgements: This study was supported by contract DAMD17-77-C-7054 from the U.S. Army Medical Research and Development Command.

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Abstract

Several serotypes of Salmonella were shown to release increased amounts of a cholera toxin-like toxin during culture in vitro with Mitomycin C. Filter sterilized culture supernatants containing the toxin caused elongation of Chinese hamster ovary cells, which could be blocked by heating the supernatants at 100°C for 15 minutes, or adding mixed gangliosides or monospecific cholera antitoxin. When Mitomycin C was not added to the Salmonella cultures, little or no detectable toxin was released. Optimal production of toxin was observed in the presence of 0.5 µg/ml of Mitomycin in shake flask cultures of CYE medium, Syncase, or peptone saline at 37°C. Meat infusion media (HI and BHI) resulted in poor toxin yield. Culture filtrates frequently could be diluted 1:8 and still result in elongation of CHO cells, but were too dilute to cause fluid accumulation in ligated intestinal loops of adult rabbits without further concentration.

Introduction

The pathogenesis of Salmonella mediated diarrheal disease has eluded virtually all investigative attempts designed to probe the pathogenic mechanisms of this notorious group of enteric pathogens. Unlike Vibrio cholerae and enterotoxigenic species of Escherichia coli, the salmonellae are noted for their invasive capacity of small intestinal epithelium. Takeuchi et al. (14), using transmission electron microscopy, carefully examined the penetration of small intestinal epithelial cells by Salmonella species. Subsequently, Giannella et al. (5) concluded that epithelial cell invasion was correlated with the capacity of Salmonella to cause loss of water and electrolytes from the intestine, culminating in diarrhea. The reason that some Salmonella infections result in a self-limited form of "food poisoning", commonly referred to as salmonellosis, while other intestinal infections with Salmonella spread to a variety of extraintestinal sites and cause an overwhelming form of "enteric fever" requiring prompt antibiotic therapy remains to be elucidated.

Salmonella, like other Gram negative bacteria, possesses heat stable endotoxin on its surface, which for many years was implicated in the loss of fluid and electrolytes from the small intestine during salmonellosis. While animals injected parenterally with endotoxin do develop diarrhea among other symptoms, oral administration of endotoxin or injection of endotoxin into the intestinal lumen fails to elicit a diarrheal response (2,15). Furthermore, the lack of diarrhea among

normal individuals, whose intestinal tract is heavily colonized with endotoxin producing strains of "normal flora" bacteria suggested that an additional virulence factor might be produced by Salmonella.

Attempts to identify an enterotoxin (or exotoxin) responsible for the diarrheal response elicited by Salmonella were unsuccessful until the independent reports by Koupal and Deibel in 1975 (8) and Sandefur and Peterson in 1976 (11). The enterotoxic principle described by Koupal and Deibel (8) and more recently by Sedlock et al. (13) was associated with the cell wall or outer-membrane fraction and caused fluid loss when administered to infant mice. The factor was destroyed at 80°C or above in 30 minutes, and Sedlock et al. (13) later reported that the factor produced positive intestinal loops in adult rabbits. Sandefur and Peterson (11) reported the isolation and partial purification of two skin permeability factors (PF's) in culture supernatants of Salmonella typhimurium. The first PF was heat stable and elicited a rapid skin permeability response, typically producing erythema and flat, soft edema at the site of intradermal injection. The skin reaction appeared as a relatively flat, blue spot when the rabbit was given an intravenous injection with pontamine sky blue dye. The second factor, referred to as the delayed PF, was heat labile and elicited an edematous, erythematous skin reaction accompanied by bluing which became maximal after 18-24 hours. This Salmonella delayed PF reaction, which is indistinguishable from that produced by cholera toxin, can be blocked by both cholera antitoxin and GM_1 ganglioside (9,12). Further studies will be necessary to determine whether this

delayed PF is the same as the enterotoxic factor isolated by Koupal and Deibel. We believe that the Salmonella delayed PF is responsible, at least in part, for the loss of fluid and electrolytes from the small intestine because 1) rabbits immunized with heat inactivated, purified cholera toxin (procholeragenoid) are protected against fluid loss when their intestinal loops are challenged with live Salmonella typhimurium (9) and 2) partially purified delayed PF will result in fluid accumulation in rabbit intestinal loops that is blocked by monospecific cholera antitoxin (J.W. Peterson-unpublished data).

Preparations exhibiting the delayed PF reaction have in previous studies required concentration and partial purification using Sephadex gel chromatography to demonstrate the skin activity (11). The requirement for such processing of culture filtrates has hampered basic studies to determine optimal conditions for delayed PF production. This report describes a reliable procedure for estimation of toxin in crude, unconcentrated filtrates of Salmonella species. A variety of cultural conditions and strains of Salmonella have been examined in an attempt to maximize toxin production and release into the culture medium. To facilitate assay of the low levels of delayed PF in the crude culture filtrates, this study was performed using the Chinese hamster ovary (CHO) cell assay of Guerrant et al (6). We have previously reported the successful use of the CHO cell assay to measure the delayed PF from partially purified Salmonella preparations and henceforth will refer to the delayed PF as Salmonella toxin (12).

Ordinarily, it is not possible to detect the heat labile Salmonella toxin in crude, unprocessed culture filtrates; however, we have found that addition of Mitomycin C to the Salmonella culture two hours after inoculation causes the release of detectable amounts of toxin from the bacterial cells. The successful use of Mitomycin C for the purpose of enhancement of toxin release was first reported by Isaacson and Moon (7) for E. coli heat-labile toxin. The initial hypothesis regarding the mechanism of Mitomycin C induced toxin release among E. coli was that it caused derepression of plasmid gene expression (7). More recently, Gemski et al. (4) reported that the drug activates a temperate phage which causes lysis and release of cellular contents. Although it remains to be determined if Mitomycin C is acting through phage lysis or plasmid gene derepression in Salmonella, the drug provides a reliable method for enhancing toxin release into crude filtrates of most Salmonella isolates.

Materials and Methods

Strains of Salmonella. Several serotypes of Salmonella enteritidis were isolated from fecal specimens by the Houston Health Department and sent to our laboratory. The cultures were maintained without further passage on trypticase soy agar slants under sterile mineral oil. The serotype identity of each numbered isolate was as follows: Sal. 10016 - serotype Javiana; Sal. 10234 - serotype Newport;

Sal. 11271 - serotype typhimurium; Sal. 9630 - serotype Newport; Sal. 9633 - serotype Newport; and Sal. 9186 - serotype Newport. Salmonella enteritidis serotype typhimurium strain 2000 was obtained from the pediatric bacteriology laboratory of the UTMB Children's Hospital and previously reported to produce the heat labile toxin (9). Salmonella enteritidis serotype typhimurium strain SR11 was kindly provided by L.J. Berry.

Bacterial Cultivation. Casaminoacids-yeast extract (CYE) broth (pH 7.0) was composed of 36 g/l Difco casamino acids, 6 g/l Difco yeast extract (YE), and 6 g/l NaCl in distilled water. Syncase was prepared with glucose (5 g/l) instead of sucrose as described by Finkelstein (3). Peptone saline was composed of 10 g/l Difco peptone and 8.5 g/l NaCl. Asparagine glucose (AG) medium was prepared with and without added lysine (5 g/l) (1). Trypticase soy broth (BBL) was prepared with and without 1% yeast extract. Heart infusion broth (Difco) and brain heart infusion broth (Difco) were used according to manufacturers directions.

Mitomycin C (Sigma lot # 96C-0361) was hydrated with sterile saline and added to Salmonella cultures two hours after inoculation. Except in the dose response study, Mitomycin C (MTC) was used at a concentration of 0.5 microgram/ml. Culture flasks were wrapped in aluminum foil to protect the MTC from light. Except where otherwise specified, 20 ml or 50 ml cultures were grown in 125 ml or 250 ml screw

top Erlenmeyer flasks, respectively, at 37°C in a gyratory shaker incubator (New Brunswick) set at 90-100 RPM for 18-20 hr. Cells were removed by centrifugation (27,000 xg) and the supernatants were filter sterilized using 0.2 μm Nalgene filter units.

CHO Cell Assay. Stock cultures of CHO-cells (12) were grown at 37°C with 4% CO_2 in F-12 medium (GIBCO) supplemented with 10% fetal calf serum and 100 units/ml of penicillin G and 100 μg/ml of streptomycin. For the CHO cell elongation tests, cells were suspended in F-12 medium containing 1% fetal calf serum and 100 units/ml of penicillin G and 100 μg/ml of streptomycin. A 0.1 ml amount of the suspension containing approximately 1000 cells was delivered to each well of microculture plates. After 3-4 hours of incubation at 37°C with 4% CO_2 ten μl volumes of toxin dilutions were added to the appropriate wells; incubation was then continued under the same conditions for 18-20 hours longer. At this time, the culture medium was decanted and the cells were fixed with methanol for 3-5 minutes. After the plates had air dried, Giemsa's stain was added to each well for 15 minutes. After washing thoroughly with tap H_2O, the plates were air dried. At least 100 cells were counted in each well and the percent of elongated cells was determined.

Results

Figure 1a illustrates the typical shape of normal CHO cells as they appear after staining with Giemsa's stain. When CHO cells are

exposed to Salmonella toxin, they appear elongated as shown in Figure 1b. The elongation effect caused by the heat labile Salmonella toxin from unconcentrated MTC filtrates is virtually identical in appearance to that caused by cholera toxin (6,12).

Figure 2 shows the striking effect of increasing amounts of Mitomycin C on two clinical isolates of Salmonella enteritidis (serotypes Newport and typhimurium). The dose response curves indicate that a concentration of 0.5 µg/ml in the culture medium is optimal for maximum release of the Salmonella toxin after 18 hours at 37°C in shake flask cultures of trypticase soy broth. The background rate of elongation observed with uninoculated trypticase soy broth was approximately 20% with or without addition of Mitomycin C; therefore, the effect of the Mitomycin C was not upon the CHO cells but upon the level of toxin production and/or release by bacteria. Similarly, the addition of Mitomycin C to crude filtrates had no effect on the activity of preformed toxin (data not shown).

Figure 3 shows composite data from a survey of clinical isolates of Salmonella for toxin production. The first frame indicates that uninoculated trypticase soy broth, both with and without 0.5µg/ml of MTC, causes a background elongation level of approximately 20%. In contrast, five out of the seven isolates examined responded by releasing up to 8 times more toxin into the culture medium at 37°C in 18 hours with the use of MTC. Two of the Salmonella strains do not appear to release increased levels of toxin under these conditions.

Interestingly, strain 2000 had been producing heat labile toxin

several months previously, but for reasons presently unknown to us has lost this ability. Recent data has shown that the elongation effect produced by strain 2000 was heat stable and not neutralized by cholera antitoxin. Strain 2000 was stored at 4°C on Trypticase soy agar slants with minimal transfer for approximately 12 months, while all clinical isolates were received 2-3 months prior to assay.

In order to characterize the factor released from Salmonella cells by Mitomycin C induction and confirm that it was the same as that described previously by our laboratory, the following study was performed. Figure 4 illustrates that crude culture filtrates from four Salmonella isolates grown at 37°C for 18 hours exhibited MTC released toxin which was heat labile, inactivated by ganglioside, and neutralized by cholera antitoxic serum. The open bars in this figure depict the CHO cell elongation caused by each of the four Salmonella filtrates, while the adjacent bars show the significant loss of activity after boiling for 15 minutes or addition of mixed gangliosides or antitoxic serum. Also shown in the figure is the background elongation caused by the uninoculated trypticase soy culture medium treated in the same manner. For comparison purposes, cholera toxin behaved in a manner similar to the Salmonella toxin. Based on these characteristics, the Mitomycin C induced Salmonella toxin is virtually identical to that previously studied in this laboratory (9) and is remarkably similar to cholera toxin.

In order to increase the yield of toxin further, a survey was performed to determine the best culture medium for optimal toxin

production in the presence of MTC at 37°C in 18 hours. Figure 5 illustrates the extent to which the filtrates from two Salmonella isolates grown in two such media could be diluted. The poorest toxin yield by both isolates was observed with brain heart infusion broth, while one of the best yields was observed with CYE medium, which is composed of casamino acids, yeast extract and NaCl. If one selects the filtrate dilution which causes 50% maximal elongation and equates this dilution reciprocal with units of toxin, the relative toxin content of several culture media can be compared. Table 1 shows that shaken cultures of CYE medium, Syncase, and peptone saline yield the highest amounts of toxin in 18 hours at 37°C. Lesser amounts of toxin were observed in asparagine glucose medium with and without lysine, trypticase soy broth with or without yeast extract, and the meat infusion broths such as heart infusion (HI) and brain heart infusion (BHI) broths.

Figure 6 summarizes an attempt to determine the effect of incubation temperature on the level of toxin produced by two strains of Salmonella growing in CYE medium at 37°C. The data indicate that the toxin is released into the medium in approximately the same amounts regardless of the incubation temperature. Strain SR11 appeared to grow very poorly at 42°C, which may account for the slightly lower amount of toxin produced at this temperature. Interestingly, there appeared also to be little effect of conditions of aeration on toxin production by the same two Salmonella strains. As shown in Figure 7, the toxin was elaborated under both aerobic and anaerobic conditions when grown in CYE medium at 37°C. Furthermore, both still and shaken cultures yielded approximately the same amount of toxin.

An attempt was made to activate Salmonella toxin present in crude filtrates using trypsin in a manner similar to the method of Rappaport et al. (10) for E. coli LT. Figure 8 indicates that the addition of trypsin one hour before or after harvest did not appreciably alter the amount of Salmonella toxin activity in filtrates of either Salmonella isolate tested. A repeat of this experiment substantiated that the toxin present in Salmonella filtrates with or without Mitomycin C could not be activated.

Discussion

The addition of Mitomycin C to cultures of Salmonella provides a reliable method for the release of heat labile, cholera-like toxin detectable using the Chinese hamster ovary cell assay. Prior to use of this technique, reliability of toxin production was poor and detection of toxin in crude, unprocessed filtrates was not possible. Despite the release of significant amounts of toxin by MTC, the actual concentration in the crude filtrates is very low and usually can be diluted out completely by making a 1:10 dilution of the filtrate. If one assumes that the Salmonella toxin has the same specific activity as cholera toxin (not yet determined), most of the MTC induced Salmonella filtrates examined thus far would contain approximately 0.01 ng/ml or less of Salmonella toxin as determined by the CHO cell assay. Compared to the larger amounts of the closely related toxins produced by Vibrio cholerae and Escherichia coli, Salmonella toxin is produced in

exceedingly low amounts using the current in vitro experimental conditions. This probably accounts for our inability to obtain fluid accumulation responses in rabbit intestinal loops after injection of the unconcentrated, MTC induced Salmonella filtrates. Likewise, we have been unable to use more convenient serological tests for cholera toxin such as Ouchterlony analysis, radioimmunoassay, and passive hemagglutination inhibition.

The basis for release of the Salmonella toxin by Mitomycin C is presently unknown, but may involve formation of lytic phage and/or plasmid gene derepression. Since we have not yet completed an analysis of the phenomenon in Salmonella, caution should be taken before concluding that strains failing to release toxin in response to Mitomycin C are truly nontoxinogenic. Such strains might possess the appropriate genetic capacity for toxin synthesis, but might lack the phage possibly needed for cell lysis and toxin release.

Using a Mitomycin C concentration of 0.5μ g/ml, a limited survey of clinical isolates of Salmonella revealed that five out of seven strains examined produced the toxin. Although the amount of toxin produced is small, it occurs with rather high frequency. The high frequency of toxin production is logical since Salmonella is virtually always associated with diarrheal disease when present in the gastrointestinal tract. In addition, one of the negative isolates, strain 2000, had been producing heat labile PF several months previously, but appeared to gradually lose this ability. The reason for this instability remains to be determined, and we are not certain whether this involves loss of some aspect of toxin release or some

genetic factor in the form of a plasmid, chromosomal gene, or temperate phage.

We have examined a number of cultural conditions (in the presence of MTC) which are conducive to toxin production. Since gangliosides inactivate the Salmonella toxin, the ganglioside content of some media (i.e. BHI) may account for their poor performance. Interestingly, other factors do not seem to influence toxin production. The amount of toxin seems to be approximately the same when cultures are grown between 25 and 42°C. In addition, oxygen tension seems to be unimportant since toxin was produced in shaken and still cultures as well as anaerobically. Since the amount of toxin released in vitro is so small, there still may be nutritional deficiencies in the artificial culture media. On the other hand, the efficiency of toxin delivery to the intestinal epithelium by the Salmonella cells in vivo may not require release of large quantitities of toxin. This is particularly pertinent since we do not yet know the relationship between toxin release and invasion of the intestinal epithelium by Salmonella. Similarly, we do not understand what triggers release of the toxin in vivo and whether this occurs before or after epithelial cell invasion. Since the toxin does appear to cause intestinal fluid loss, however, there is apparently a sufficient amount of toxin released in vivo for its action to be evident.

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Figure Legends

Figure 1. Frame A illustrates normal CHO cells stained with Giemsa's stain. Frame B shows stained CHO cells after treatment with heat labile Salmonella toxin in an unconcentrated MTC filtrate.

Figure 2. Dose response curves of two Salmonella species in response to Mitomycin C showing enhanced release of the heat labile toxin as detected by CHO cell elongation.

Figure 3. Survey of several clinical isolates of Salmonella for toxin production. Curves depicting greater CHO cell elongation in response to Mitomycin C are marked + MTC.

Figure 4. Characterization of the toxin in filtrates released by several isolates of Salmonella grown in the presence of Mitomycin C.

Figure 5. Dilution curves of CYE and BHI media after culture with two clinical isolates of Salmonella.

Figure 6. Effect of temperature on release of toxin by two clinical isolates of Salmonella.

Figure 7. Effect of oxygen tension on release of toxin by two clinical isolates of Salmonella.

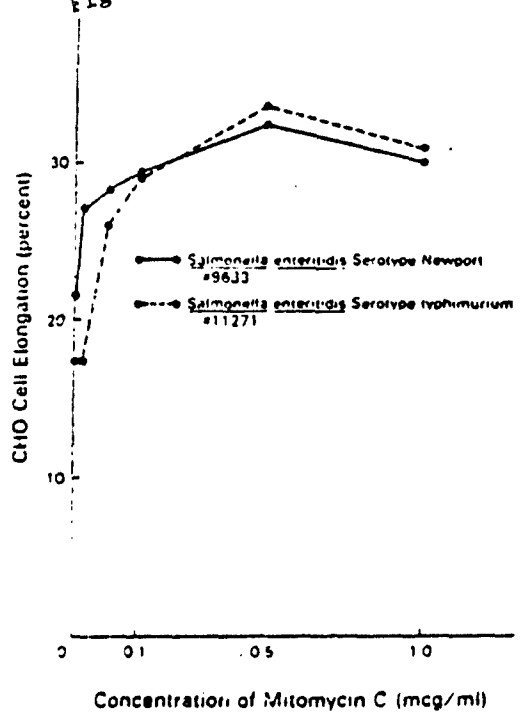
Figure 8. Attempt to activate Salmonella toxin by treatment with trypsin.

Table Legend

Table 1. Effect of culture medium on release of toxin by two clinical isolates of Salmonella.

Figure 1

Figure 2



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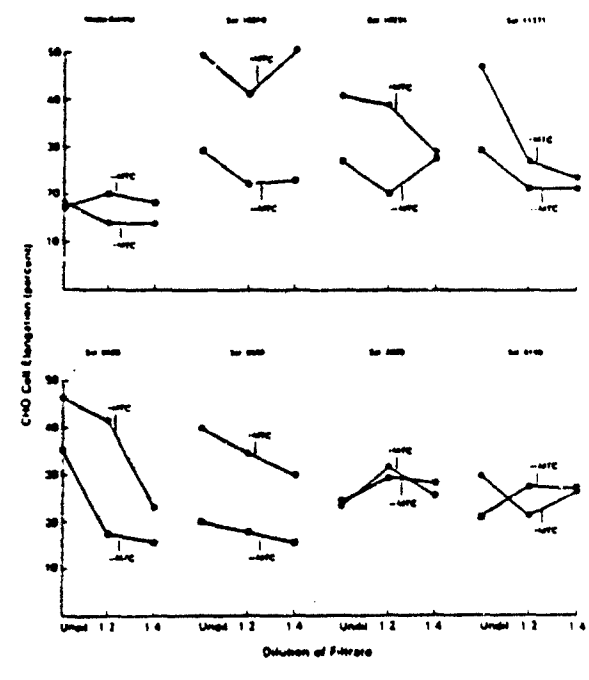

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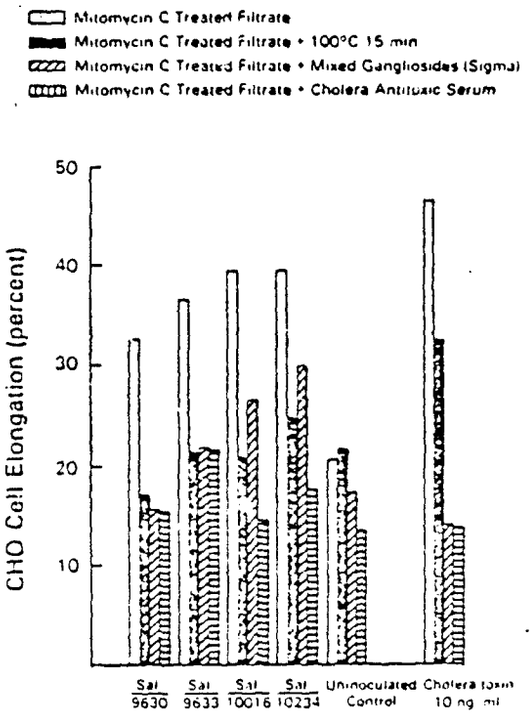

Figure 5

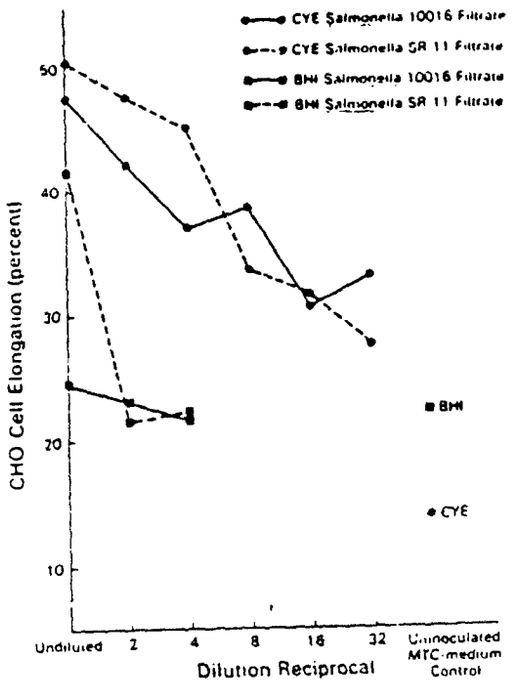

Figure 6

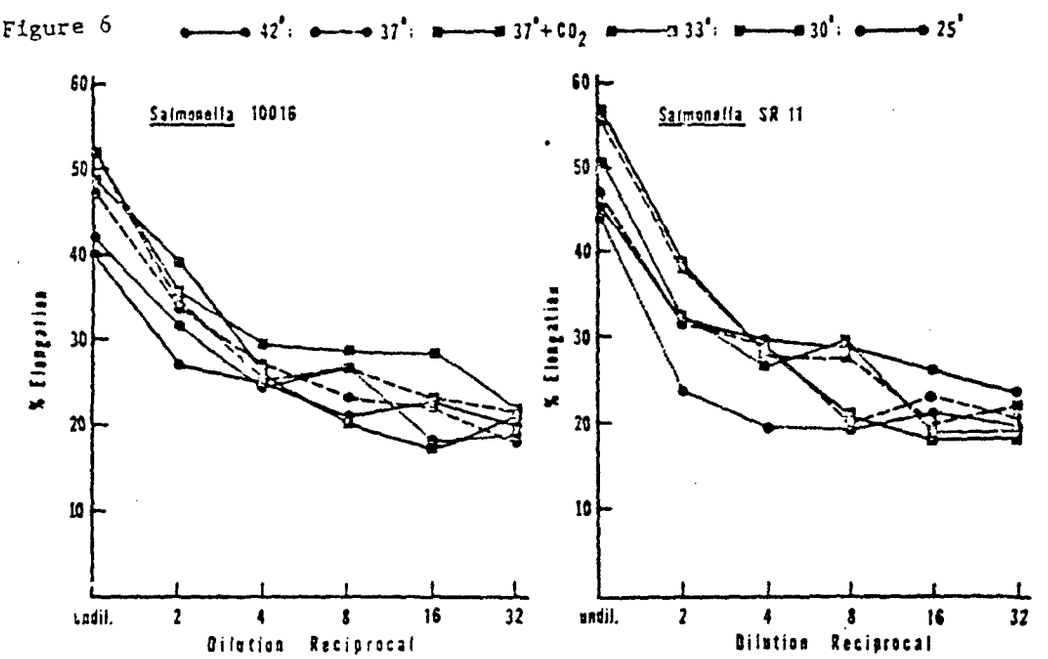

Figure 7

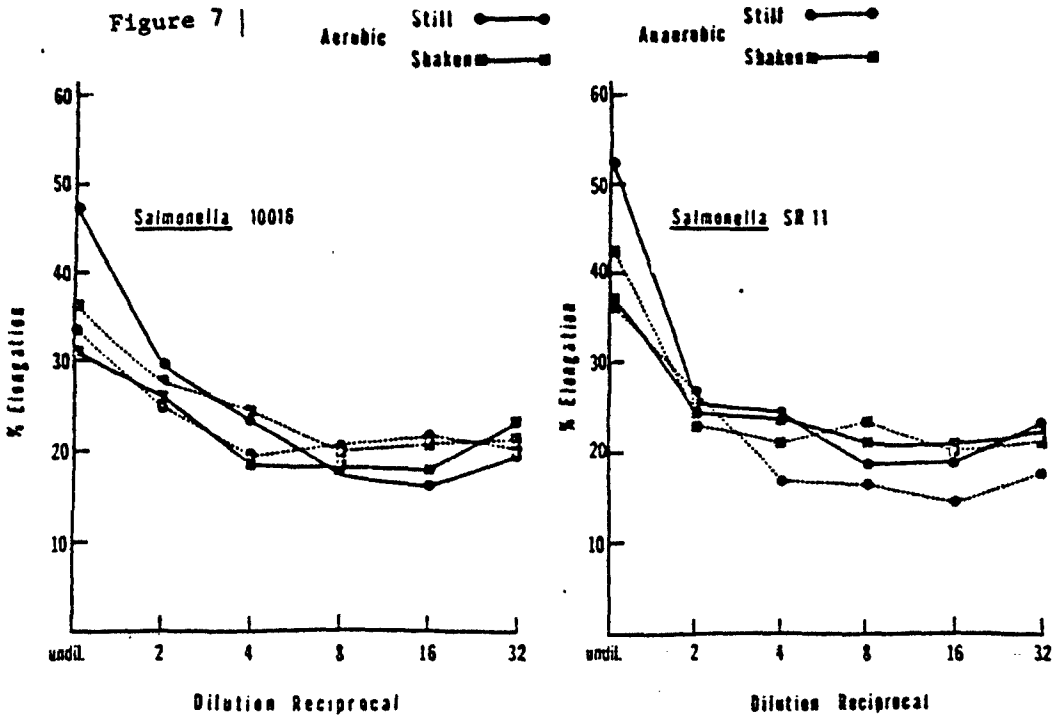

Figure 8

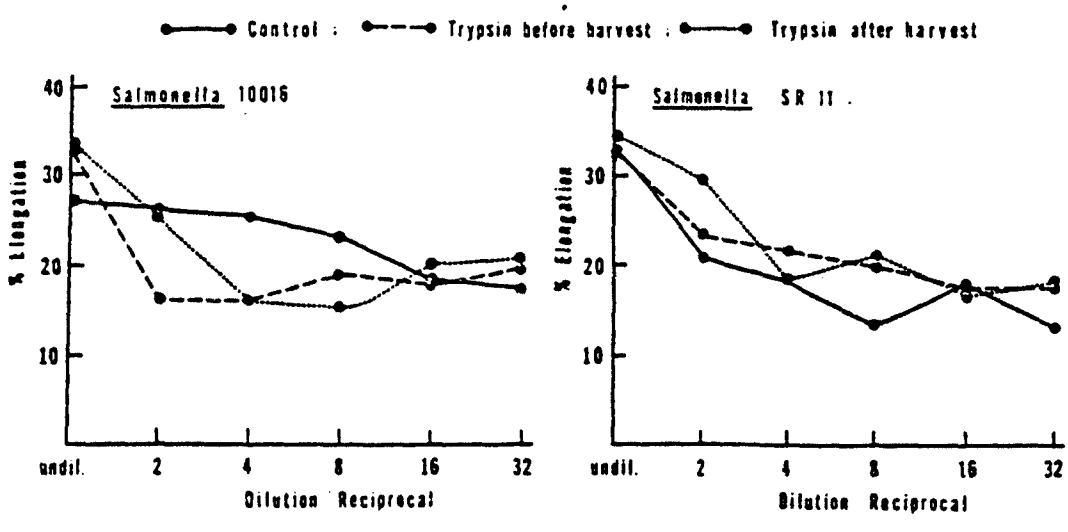

Table 1

CULTURE MEDIA SURVEY

Culture Media	*Salmonella enteritidis* serotype Javiana #10016 C H O Cell Factor (Units/10 µl)	*Salmonella typhimurium* SR11 C H O Cell Factor (Units/10 µl)
CYE	32	16
Syncase	12	36
Peptone saline	21	24
AGL	11	2.5
TSB	8	6
TSB+1%YE	3	5
AG	4	2
HI	1.75	2
BHI	1.50	1

Unit: The reciprocal of the culture filtrate dilution (10 µl) which will result in 50% maximal elongation of CHO cells.

II. B.

Enterotoxic Activity of Salmonella Delayed Permeability Factor

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ABSTRACT

A partially purified preparation of the delayed skin permeability factor from Salmonella typhimurium exhibited enterotoxic activity in ligated intestinal loops of adult rabbits. The fluid accumulation response was blocked by heating the toxin preparation at 100°C for 10 minutes or by addition of monospecific cholera antitoxin. In addition, rabbits immunized with procholeragenoid were protected against fluid loss following live cell challenge with live Salmonella typhimurium. Antisera to cholera toxin or Salmonella toxin neutralized cholera toxin as well as Salmonella toxin.

A permeability factor (PF) in filtrates of Salmonella typhimurium was recently reported to be similar in biological activity and antigenic characteristics to cholera toxin (10, 13). This exotoxin appeared to be a heat labile protein of approximately 90,000 d that was neutralized by combination with monospecific cholera antitoxin or G_{M1} ganglioside. Partially purified preparations of the Salmonella toxin, but not crude filtrates, elicited an erythematous, edematous response after intradermal injection in adult rabbits. Later, the toxin preparations were shown to elongate Chinese hamster ovary cells (15). Initial attempts to demonstrate that Salmonella filtrates and toxin preparations possessed the capacity to induce a fluid accumulation response in adult rabbits and mice were unsuccessful (10). In the present study, we have demonstrated that concentrated, isotonic preparations of the Salmonella delayed PF elicit the loss of fluid from the small intestine of adult rabbits. Furthermore, rabbits immunized with procholeragenoid exhibited 14-28 fold protection against fluid accumulation in intestinal loops challenged with Salmonella typhimurium.

Preparation 1 and 2 of Salmonella toxin were partially purified from fermenter culture supernatants. Salmonella typhimurium strain 2000, grown in 12 liters of trypticase soy broth (BBL), was incubated at 37°C for 18 hours with 5 liters/min aeration. Cells were removed by centrifugation of cultures in 1 liter bottles at 6975 x g. The toxin in the supernatant was precipitated by addition of 2.55g/l of sodium metaphosphate and lowering the pH to 4.6 with concentrated HCl (12). The precipitate was collected by filtration through Millipore prefilter

pads in a Buchner funnel and eluted with 0.01 M potassium phosphate buffer (pH 6.5). After dialysis in the same buffer, the toxin preparation was pumped through a 2.5 x 25 cm column of Affigel 102 (Biorad) equilibrated with 0.01 M phosphate buffer (pH 6.5). Elution of toxin from the column was achieved with a gradient of phosphate buffer up to 0.5 M. Phosphate buffer was removed by dialysis against 0.05 M Tris buffer (4). Preparation II was further chromatographed through a 2.5 x 100 cm column of Sephadex G100. After each step biological activity was located by skin testing in adult rabbits (13).

Procholeragenoid was prepared from purified cholera toxin as described previously (3,7). Glutaraldehyde toxoid lot #20101 was prepared by Rappaport et al. (11). Rabbits were immunized with three 200 µg intramuscular injections of either toxoid administered at two week intervals. Live cell challenge was scheduled two weeks after the last injection.

Intestinal loops in adult New Zealand rabbits were prepared as described previously (2,8). Briefly, a series of eight intestinal loops about 10 cm apart were made with surgical silk. Interspaces 2 cm in length were tied between each loop through which injections of Salmonella toxin or live Salmonella typhimurium (strains 986 or 2000) were made. The proximal and distal loops always received 1.0 ml of PBSG as a control when live cells were used for challenge. Control loops were injected with 0.05 M Tris buffer (4) when toxin preparations were used for challenge. Live cell challenge doses ranged from 10^3 to 10^8 viable cells/ml, and the dose of cells causing accumulation of 1 ml of fluid per cm of intestinal loop was selected as the ED_{50}.

Permeability factor activity of Salmonella toxin preparations was determined as described previously (1) and was expressed as the dilution of toxin eliciting a bluing zone of 4 mm (BD_4). Neutralization of skin permeability factor activity was accomplished by incubation of the Salmonella toxin with either cholera antitoxin dilutions or antiserum to partially purified Salmonella toxin prior to skin testing (1, 10).

Antiserum to the partially purified Salmonella toxin was prepared by immunization of four adult New Zealand white rabbits with the toxin. The specific activity of the Salmonella toxin preparation used for immunization was 32 $BD_4/\mu g$, and rabbits received doses of 175 µg of protein emulsified in Freund's complete adjuvant every four weeks. Blood samples were obtained prior to immunization and every two weeks thereafter. Serum was collected and stored at -20°C. Antiserum to purified cholera toxin was prepared as described previously (5, 9).

Live cell agglutination tests for antibodies to Salmonella were performed on selected sera from rabbits subjected to live cell challenge. Salmonella typhimurium strain SR11 was grown on heart infusion agar plates and suspended in phosphate buffered saline with gelatin (16). The cell suspension was adjusted to a reading of 170 on a Klett-Summerson colorimeter. This corresponded to a viable cell count of 2×10^9 CFU/ml. Two fold dilutions of each heat inactivated serum sample (0.3 ml) were prepared and 0.3 ml of a 1:5 dilution of the cell suspension was added. After incubation at 37°C for 2 hours, the assay tubes were refrigerated overnight. Agglutination was assessed by examining the extent of agglutination and results were expressed as the reciprocal of the highest dilution yielding agglutination of the bacterial cells.

Protection of rabbits against fluid accumulation from intestinal challenge with Salmonella by parenteral immunization with procholeragenoid was initially reported by Peterson and Sandefur (10, 14). Table 1 presents data that confirms the efficacy of the cross protection. In both groups of procholeragenoid immunized rabbits, 14-29 fold protection against Salmonella challenge was observed. Rabbits immunized with glutaraldehyde toxoid failed to show any protection (data not shown).

Demonstration of cross protection as described above provides indirect evidence that the cholera toxin-like exotoxin present in filtrates of Salmonella species is an enterotoxin. The data in Table 2 indicates that concentrated preparations of Salmonella toxin possess enterotoxic activity. A dose response curve is not presented since only limited quantities of the toxin concentrate were available; however, greatest enterotoxic activity was obtained in the toxin preparation exhibiting the most skin permeability factor activity. Table 3 further indicates that the enterotoxic response, as well as the skin PF response, could be blocked by heating the toxin preparation at 100°C for 10 minutes or by preincubation with 0.5 ml of monospecific cholera antitoxin (410 units/ml). When crude supernatants of Salmonella cultures, grown with or without Mitomycin C (6), were injected into intestinal loops in a similar fashion, no fluid accumulated. Therefore, the level of the enterotoxin in culture filtrates is too low to detect. This is confirmed by the low level of CHO cell activity found in crude culture filtrates (6).

Variation in skin and intestinal loop responses in adult rabbits appears to be a major problem in use of the animal models for the study of Salmonella toxin. For several years we have encountered variation among rabbits in response to Salmonella toxin. Figure 1 illustrates a typical example of the variation in skin PF responses of two rabbits randomly injected with identical preparations of toxin. Note the overall superior response of the rabbit on the left compared to the rabbit on the right. Usually by injecting pairs of animals, this problem can be reduced.

Table 4 shows that recently we have encountered more than the average amount of variation in rabbits challenged with Salmonella strains. Slightly more than one half of the rabbits examined in this recent study failed to respond with fluid accumulation. The innate resistance of this group of animals may have resulted from prior exposure of the population to Salmonella antigens. As shown in Table 4, there was a tendency for animals possessing higher Salmonella agglutination titers to fail to respond to intestinal loop challenge with several strains of Salmonella. Although this group of rabbits appeared to be more resistant to Salmonella than normal, we feel that prior antigenic exposure of rabbits during Salmonella infections could account for the variation in skin and intestinal loop responses seen in previous studies.

Table 5 shows the rise in cholera antitoxin titer in the sera of two rabbits immunized with partially purified Salmonella toxin. The level of antibody capable of neutralizing cholera toxin rose to

detectable levels following immunization, although the final antitoxin level was low. Two other rabbits failed to develop neutralizing antibodies to either toxin. Since these sera also neutralized the homologous Salmonella toxin, and antisera to cholera toxin neutralized Salmonella toxin, the two toxins appear to possess remarkably similar antigenic structures.

In the present study we have shown that the Salmonella delayed PF possesses enterotoxic activity in adult rabbits. The level of toxin in crude culture filtrates was not detectable using the intestinal loop assay and concentrated, partially purified preparations had to be used. Confirmation of heterologous protection against fluid loss in response to intestinal challenge with Salmonella was achieved by immunization of rabbits with procholeragenoid. Immunization of rabbits with partially purified Salmonella toxin resulted in the formation of antibodies that could neutralize both cholera toxin and Salmonella toxin. This intriguing antigenic relationship will be difficult to study further until techniques of increasing toxin yield are developed. The problem of variation using the rabbit as an experimental animal was noted. Since Salmonella frequently infect animal populations, we have tended to use the CHO cell assay (6) whenever possible; however, improvements in the animal model are needed to characterize the effect of Salmonella toxin on the intestinal tract and assess its significance.

Table and Figure Legends

Table 1. Protection of rabbit intestinal loops against fluid accumulation in response to challenge with Salmonella typhimurium by immunization with procholeragenoid.

Table 2. Biological activity of partially purified Salmonella toxin in rabbit intestinal loops and skin.

Table 3. Loss of enterotoxic and skin permeability activity of Salmonella toxin after heating (100°C 10 min) and incubation with monospecific cholera antitoxin.

Table 4. Relationship of normal rabbit intestinal loop responses to serum agglutination titers.

Table 5. Rise in cholera antitoxin titer in sera of rabbits immunized with partially purified Salmonella toxin.

Figure 1. Comparison of the variation in responses of two rabbits subjected to skin test with Salmonella toxin. The induration and bluing responses of the rabbit on the left are markedly superior to those of the rabbit on the right.

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Table 1

Experiment	Group	Live Cell Challenge of Intestinal Loops ED_{50} Value	Protection Factor
Experiment 1 Loop Challenge with *Sal. typhimurium* strain 986	Procholeragenoid n = 13	8.1×10^7	28.9
	Control n = 17	2.8×10^6	0
Experiment 2 Loop Challenge with *Sal. typhimurium* strain 2000	Procholeragenoid n = 7	2.3×10^9	14.4
	Control n = 8	1.6×10^8	0

Table 2

Sample	Skin Test Potency BD_4/ml	Intestinal Loop Response* ml/cm	Protein Concentration µg/ml
Salmonella Toxin Prep. I	331	0.92	70
Salmonella Toxin Prep. II	11,200	1.91	353

* Dose = 0.5 ml

Table 3

Sample	Fluid Accumulation ml/cm	Skin Test Potency BD_4
Salmonella Toxin* Preparation II	2.1	11,200
Heated *Salmonella* Toxin 100°C 10 min	0.6	0
Salmonella Toxin +0.5 ml cholera antitoxic serum	0.6	0
0.01 MPO_4	0.24	0

*Protein Conc. = 350 µg/ml

Table 4

Rabbit No.	Loop Fluid Response*	Live Cell Agglutination Titer (Dil Reciprocal)
1	-	16
2	-	8
3	-	8
4	-	8
5	-	8
6	± slightly positive	8
7	-	4
8	-	4
9	+	4
10	+	4
11	-	2
12	+	2
13	-	2
14	+	<undil

*Response to Loop Challenge with Salmonella W118-2, 2000, or SR11 (10^9 cells/loop)

Table 5

Immunization Time (weeks)	Cholera Antitoxin Titer PF Neutralization (units/ml*) Rabbit #2	Rabbit #4
0	<0.69	<0.69
2	<0.69	0.87
4	1.8	1.4
6	<2.8	<0.69
8	3.5	<0.69
10	4.3	0.69
12	15.8	3.5
14	12.0	3.5

*Based on comparison with the Swiss Serum and Vaccine Institute reference serum.

III. Additional Results (not reported in manuscripts)

A. Salmonella Heat Labile Toxin and Systemic Infection. Previous data in our laboratory has indicated that the cholera toxin-like toxin present in culture filtrates of Salmonella species may be responsible in part for the loss of fluid and electrolytes during salmonellosis. This conclusion was based upon the toxin's enterotoxic activity demonstrable in rabbit intestinal loops and the protection of rabbit intestinal loops against challenge with live Salmonella following immunization with procholeragenoid. Although more investigations are necessary to determine the precise relationship of this toxin to diarrheal disease, we have often speculated that it might play some role in systemic Salmonella infections. While performing surveys of Salmonella serotypes for production of the toxin, we observed that Salmonella typhimurium strain SR11 elaborated toxin that elongated CHO cells, while S. typhimurium strain RIA did not. Interestingly, strain SR11 was said to be a highly virulent strain for mice, while strain RIA was weakly virulent (personal communication- L.J. Berry). We have recently determined that the LD_{50} of strain SR11 is 173 cells. Additional LD_{50} determinations on selected toxinogenic and nontoxinogenic Salmonella strains are in progress to examine this phenomenon further.

In addition, an experiment is in progress to determine if adult mice immunized with either 10 µg or 1 µg of procholeragenoid differ from nonimmunized mice in response to intraperitoneal challenge with Salmonella typhimurium SR11. Two weeks after primary immunization, the three groups of mice were challenged with intraperitoneal doses ranging

from 10^0-10^5 Salmonella (n=10 mice/dose). The mice will be held for 21 days, but it appears thus far that the LD_{50} will be approximately the same for each of the procholeragenoid immunized and nonimmunized groups. Thus, in this systemic infection model, there appears to be no apparent involvement of the cholera toxin-like toxin. Before abandoning the hypothesis of an extraintestinal involvement of this toxin, an attempt will be made to correlate the LD_{50} of Salmonella strains with their capacity to elaborate the heat labile toxin.

B. Intestinal Loop Studies. Problems with the ligated intestinal loop assay have been encountered recently. We have observed an increased occurrence of false positive loops in rabbits purchased from our supplier. These false positive reactions are due apparently to coccidial parasite infections. An occasional animal had a caecum that was filled with fluid and intestinal tissues were heavily infected with these encysted forms of the parasite. We tried treatment of already purchased animals with sulfa drugs, but subsequent challenge results were basically unchanged with regard to false positive reactions. Besides the false positive reactions, which occurrred predominantly at the ileal end, rabbits purchased recently from our regular supplier and one other source appeared to be innately resistant to challenge with doses up to 10^{10} cells and often failed to respond with fluid accumulation. Many of the rabbits which failed to yield fluid accumulation also possessed live cell agglutination titers to Salmonella typhimurium. Therefore, the rabbit population has become unsatisfactory and has hindered studies involving this model. We have postponed further use of the rabbit loop assay to study toxin until the problem

with animal population is solved. At that time we will attempt to determine if cholera antitoxic serum, gangliosides, or cholera toxin B subunit will block fluid accumulation when injected into intestinal loops challenged with Salmonella.

C. Suckling Mouse Assay. The suckling mouse assay has been used successfully to detect the heat stable toxin of Escherichia coli. We have fed several preparations of Salmonella heat labile toxin to groups of 3-5 day old mice via a 20 gauge cannula. The pontamine sky blue dye mixed with the toxin preparations entered the intestinal tract, but the intestine/carcass weight ratios of control mice and toxin treated mice were routinely equal to approximately 0.05. The assays were performed over a four hour period, but in one experiment, the mice were held for 18 hr at 37°C prior to autopsy. Again there was no difference between control and toxin fed groups. Therefore, we do not believe that the Salmonella toxin will yield a positive response in this model, despite the fact that samples were tested that were known to be positive in the CHO cell assay, the skin test, and rabbit intestinal loops.

D. Examination of Alternate Methods of Toxin Release. Several procedures were examined in an attempt to cause release of heat labile toxin from Salmonella cells. Laboratory techniques such as growth in the presence of lincomycin (104) or incubation with polymyxin B (103) have been reported to increase filtrate levels of E. coli heat labile toxin. Treatment of cells with EDTA (105) has been reported to release cell surface components and sonication cells should lyse cells, releasing intracellular products. After examining each of the above techniques,

we concluded that there was no appreciable increase in release of toxin from the cells.

E. Effect of Salmonella Heat Labile Toxin on Adrenal Cells. We would like to report that two samples of partially purified Salmonella heat labile toxin were sent to Dr. Sam Donta to determine if the toxin exhibited activity in adrenal cell cultures. He determined that adrenal cells exhibited morphologic changes (cell rounding) and steroidogenesis analogous to that of cholera toxin. Furthermore, the effects could be blocked by cholera antitoxin. Further collaborative studies have been discussed and are planned for the next project period.

F. Heat Stable CHO Cell Elongation Factor. While performing CHO cell assays on crude culture filtrates of a number of Salmonella species to detect the heat labile toxin, we have noticed that occasionally some of the elongation activity of culture filtrates is not completely destroyed at 100° C in 15 minutes. Within the last few days, for reasons that we do not yet understand, the capacity of our Salmonella strains to produce heat labile toxin seems to be diminished, even in cultures treated with Mitomycin C. This is reminiscent of the previous project period, in which we reported the loss of heat labile, delayed skin PF from Salmonella strain 986 and 2000. Although we are unaware of any cultural condition that is different from conditions used previously there could be a subtle change such as culture medium lot number. We are presently in the process of examining cultures stored under liquid nitrogen compared to the bulk of our strains stored on TSB agar slants under sterile mineral. Some cultures are also stored lyophilized.

Since so many strains seem to exhibit the same phenomenon, we suspect that this recent technical problem is not due to bacterial mutation but perhaps is related to susceptibility of the CHO cell strain to the toxin. We did encounter problems of viability with our CHO cell cultures, but were able to get the cultures growing again. This process may have selected for a population of cells that are resistant to the heat labile toxin. Perhaps this cell population may have fewer ganglioside receptors on their surface or may have a modified adenylate cyclase system. One other possibility is that there may be a problem with the phosphodiesterase inhibitor (MIX) used in our CHO cell assay. The bottle used in our lab for several years is empty to the extent of being scraped to remove residual chemical and has been frozen and thawed countless times. If this phosphodiesterase inhibitor were inactive, the CHO cell assay would be grossly less sensitive for detection of the heat labile toxins. We are presently pursuing this possibility.

The apparent absence of the heat labile toxin in some culture filtrates has revealed the presence of another interesting substance present in the culture filtrates. The factor can be diluted out by a 2-8 fold dilution, but it does cause elongation of CHO cells and is heat stable. The heat stable CHO cell factor is not neutralized by cholera antitoxin, which allows us to distinguish it from the heat labile, cholera toxin-like toxin. We are unsure of the nature and/or significance of this heat stable, CHO cell factor. We can speculate that the activity is due to 1) the presence of a different toxin capable of stimulating CHO cells but with different heat stability and antigenic characteristics, 2) adsorption of the heat labile, cholera toxin-like

toxin to a heat stable moiety present in crude culture filtrates (i.e., somatic antigen) making the heat labile toxin appear heat stable and interfering with its neutralization by cholera antitoxin, or 3) the presence of low levels of bacterial cyclic AMP, which would be heat stable and not neutralized by cholera antitoxin. We are currently approaching the problem with consideration of each of these possibilities. By adding cyclic AMP binding protein or phosphodiesterase to crude culture filtrates, it may be possible to identify bacterial cyclic AMP.

G. Production of Heat Labile Toxin by Salmonella Strains with Characterized Intestinal Virulence. Seven isolates of Salmonella were selected because their capacity to invade and cause loss of fluid and electrolytes from the intestinal lumen was well characterized. Some strains possessed the ability to invade intestinal epithelium and cause fluid accumulation, while others could neither invade nor cause fluid accumulation. Two strains could invade the intestinal epithelium, but did not cause accumulation of fluid in intestinal loops. Each of the strains were grown in CYE medium at 37°C for 18 hrs in small shake flask cultures. Bacteria were removed by centrifugation in sterile centrifuge tubes and supernatants were sterilized by filtration in 0.2 m Nalgene filter units. Sterile filtrates were stored at 4°C in sterile plastic tubes (Falcon). Toxin content of the filtrates was assayed in CHO cells within 1-2 days. The CHO cell elongation effect or positive filtrates was blocked by heating the samples 100°C for 10 minutes and by a 1:100 dilution of the preimmunization serum.

Table 1 designates the Salmonella strains that produced heat labile toxin. The first group of strains that were invasive and caused fluid accumulation all elaborated the cholera toxin-like toxin. Strain SL 1C27 was invasive but did not produce toxin or cause fluid accumulation. Some cultures of Salmonella strain 9 SR2 were observed to elaborate toxin, but it was known to be noninvasive and failed to cause fluid accumulation. The Thax-1 strain of Salmonella was nontoxigenic, noninvasive, and failed to cause fluid accumulation. The data indicate rather convincingly that both toxin production and invasion are correlated with fluid accumulation. The only exception that did not

seem to support this conclusion was LT7, which was toxigenic and invasive but not cause fluid accumulation; however, it may be defective in some other virulence factor. We believe that these data provide additional support for the working hypothesis that the Salmonella heat labile toxin is responsible in part for the loss of fluid and electrolytes in salmonellosis. It does appear that both cell invasion and toxin production are required, which distinguishes the pathogenesis of this diarrheal disease from cholera and other toxin mediated diarrheal diseases.

Table 1

Correlation of *Salmonella* Heat Labile Toxin Production with Invasion and Fluid Accumulation Capacity

Salmonella Strain[5]	Toxin[1]	Invasion[2]	Fluid Accumulation[3]
TML R66	+	+	+
W 118-2	+	+	+
M 206	±[4]	+	+
LT7	+	+	-
SL 1027	-	+	-
9 SR2	±[4]	-	-
Thax	-	-	-

[1]Heat labile toxin elongating CHO cells and neutralized by cholera antitoxin but not preimmunization serum

[2]Epithelial cell invasion - Giannella *et al.* (23)

[3]Intestinal loop response - Giannella *et al.* (23)

[4]Some culture filtrates have been positive and some have been negative

[5]*Salmonella* isolates were provided by Dr. S.B. Formal