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Quantitative Analysis and Partial Characterization of Cytotoxin Production by *Salmonella* Strains

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The pathogenesis of the wide-spectrum human disease caused by *Salmonella* species is poorly understood. Cytotoxin production by other enteric pathogens has been increasingly investigated recently, and data are accumulating regarding the role of cytotoxins in enteric infections and hemolytic uremic syndrome. We studied the cytotoxic activity of 131 *Salmonella* strains of the major serotypes, including 94 strains of *Salmonella enteritidis*, 12 strains of *Salmonella typhi*, and 25 strains of *Salmonella choleraesuis*. Cytotoxicity was quantitatively determined in sonic extracts by a [^{3}H]thymidine-labeled HeLa cell assay. All *Salmonella* strains examined showed some degree of cytotoxic activity. The geometric means ± standard deviations of the amounts of cytotoxin produced (50% cytotoxic dose per milligram of bacterial protein) were 27 ± 2 for *S. typhi*, 65 ± 2 for *S. enteritidis*, and 117 ± 2 for *S. choleraesuis*. Analysis of variance showed that the differences in cytotoxin production by the three species were significant ($P < 0.001$). No significant differences were found between stool isolates and invasive strains of the same species. Neutralization studies showed that the cytotoxins produced by all *Salmonella* strains were immunologically distinct from Shiga toxin and the closely related Shiga-like toxins produced by *Escherichia coli*. DNA hybridization studies with DNA probes for Shiga-like toxins of types I and II showed no hybridization. In each species the cytotoxin was heat labile and sensitive to trypsin treatment, which indicated that its active component was probably protein in nature. Upon ultrafiltration with Amicon membranes and gel filtration chromatography, cytotoxic activity was found in the molecular weight range of 56,000 to 78,000. Our findings indicate that salmonellae produce cytotoxin(s) that may play a role in the manifestations of the various species.

Salmonellosis continues to be an important medical problem in the United States. The number of human *Salmonella* isolates reported to the Centers for Disease Control has been increasing steadily since 1970 (7). In recent years approximately 40,000 isolates have been reported annually (5, 6), with a true incidence estimated at 1.9×10^6 *Salmonella* infections per year (7). These organisms cause a wide spectrum of human disease, ranging from self-limited watery diarrhea to inflammatory diarrhea, and sometimes bacteremia, enteric fever, and focal extraintestinal infections (10, 34). The mechanisms involved in these infections are poorly understood. It has been shown that 55% (22) to 72% (16) of *Salmonella* isolates produce heat-labile enterotoxin that is similar to cholera toxin (12, 30, 35). Some strains produced heat-stable enterotoxin (16). However, enterotoxin production does not explain all of the features of *Salmonella* infections, which include inflammatory diarrhea (34), invasiveness and damage to intestinal mucosa (13, 32, 36), and invasion of HeLa cells (27). Wallis et al. found no correlation between enterotoxigenicity in vitro and the ability of the organisms to produce disease in vivo (37).

Cytotoxin production by enteric pathogens has been increasingly investigated in recent years. These toxins (also referred to as verotoxins by those who assay cytotoxic activity in Vero cells [19]) are defined by their ability to kill mammalian cells. Shiga toxin, produced by *Shigella* strains (mainly *Shigella dysenteriae* serotype 1) (3), and the closely related Shiga-like toxins (SLTs) produced by *Escherichia coli* (8, 24, 28) are well characterized. On the basis of neutralization studies, SLTs are currently divided into two groups, SLT type I and SLT type II (SLT-I and SLT-II); only SLT-I is neutralized by antibodies to Shiga toxin (28). Cytotoxin production has also been demonstrated in *Campylobacter* sp. (15), *Clostridium difficile* (4), and other bacteria (28). Evidence is evolving regarding the role of cytotoxins in the manifestations of *Shigella* spp. (31), *E. coli* (8, 29), and *C. difficile* (4) and the major pathogenic role of Shiga toxin and SLTs in hemolytic uremic syndrome (14, 17).

Limited data are available concerning cytotoxin production by *Salmonella* strains. Cytotoxin production has been demonstrated by O'Brien et al. in one *Salmonella typhimurium* strain (29), by Ketyi et al. in three *Salmonella enteritidis* strains (18), and by Baloda et al. in two *S. enteritidis* strains (2). Further studies have shown that *Salmonella* cytotoxin inhibits protein synthesis (20, 21), as do many other cytotoxins (28). In this communication, we describe a comprehensive analysis, using a quantitative radiolabeled assay described previously (3, 8, 31), of cytotoxin production by 131 *Salmonella* strains representing major serotypes from various sources. Because Shiga toxin, SLT-I, and SLT-II are emerging as the most important, clinically relevant cytotoxins produced by members of the family *Enterobacteriaceae*, we examined their genetic and immunologic relatedness to *Salmonella* cytotoxins. In addition, we partially characterized the toxic activity and attempted to explore its role in the pathogenesis of salmonellosis.

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TABLE 1. Species and sources of 131 *Salmonella* strains examined for cytotoxicity

Salmonella species	Source	No. of strains
S. typhi[a]	Stool	5
	Blood	7
S. enteritidis[a]	Stool	83
	Blood	5
	Cerebrospinal fluid	3
	Urine	1
	Unknown	2
S. choleraesuis[b]	Stool	3
	Extraintestinal	14
	Unknown	8

[a] All human isolates.
[b] Ten isolates from humans and twenty-five from swine.

MATERIALS AND METHODS

Bacterial strains. A total of 131 *Salmonella* strains of three species were isolated from both intestinal and extraintestinal infections (Table 1). They were derived from a variety of sources. Nineteen *S. enteritidis* strains and one *Salmonella typhi* strain were obtained from the microbiologic laboratory at our hospital, and seven *S. enteritidis* strains were obtained from studies at our day-care centers. Fifty-six *S. enteritidis* strains and six *S. typhi* strains were provided by Anne Doggest, Houston City Health Department. Edward Mason (Texas Children's Hospital, Houston) provided six *S. enteritidis* strains and five *S. typhi* strains. V. Feinstein (M. D. Anderson Hospital and Tumor Institute, Houston) provided six *S. enteritidis* strains. All of these strains were isolated from humans. S. Gibson (Texas Department of Health, Austin) provided 10 human isolates of *Salmonella choleraesuis*, and 15 *S. choleraesuis* isolates from swine were provided by the National Animal Disease Center, Ames, Iowa. Control strains consisted of *Shigella dysenteriae* serotype 1 strain 60R (which produces Shiga toxin), *E. coli* serotype O26:H11 strain H30 (which produces SLT-I), *E. coli* C600 (933w) (which produces SLT-II), and *E. coli* C600 (a non-cytotoxin-producing strain).

Preparation of bacterial toxins. Bacteria were recovered for study by streaking on charcoal-yeast extract agar plates; culture purity was verified by streaking the cells simultaneously on MacConkey and blood agar plates incubated overnight at 37°C. Bacteria were then inoculated into 10 ml of iron-depleted syncase broth (3), incubated for 48 h at 37°C with shaking at 200 rpm, and harvested by centrifugation (10,000 × *g* for 10 min). The pellet was washed with 10 mM phosphate buffer (pH 7.4), suspended in phosphate buffer, and lysed by sonication (8). Cell debris was removed by centrifugation (10,000 × *g* for 20 min), and the sonic extract was sterilized by filtration (pore size, 0.22 μm). The protein concentration was determined by a protein assay (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as a standard. Purified Shiga toxin was prepared as described previously (3, 31).

Preparation of immune serum. Rabbits were repeatedly injected with 100 μg of purified, formaldehyde-treated Shiga toxin in complete Freund adjuvant (3) to obtain an immune serum which neutralized both Shiga toxin and SLT-I. Rabbit immune serum against SLT-II was kindly provided by A. D. O'Brien (Uniformed Services University of the Health Sciences, Bethesda, Md.). Nonimmune serum, obtained from unimmunized rabbits, did not neutralize Shiga toxin, SLT-I, or SLT-II.

HeLa cell cytotoxicity assay. Cytotoxicity was quantitatively determined by using a radiolabeled HeLa cell assay as previously described (8, 31). Briefly, 96-well plates were inoculated with 50 μl of serial dilutions of the cell-free toxin. Medium control wells inoculated with 50 μl of Eagle minimal essential medium (Hazleton Research Products, Denver, Pa.) were included on each plate. Seven-day-old HeLa cells were suspended in Eagle minimal essential medium with Eagle salts (Hazleton), 10% fetal calf serum, 2 mM L-glutamine, and [^{3}H]thymidine (2 Ci/ml; ICN Radiochemicals, Irvine, Calif.) and then added to each well (5×10^5 cells per ml, 100 μl per well). After overnight incubation at 37°C with 5% CO_2, the wells were washed with 10 mM phosphate buffer to remove unincorporated [^{3}H]thymidine and detached cells. Viable cells were then lysed with 1 N KOH, transferred to liquid scintillation counting solution, and counted for radioactivity (3). HeLa cell survival was determined by dividing the count in toxin wells by the count of the medium control wells (to which no toxin was added). The amount of toxin that killed 50% of the HeLa cells (50% cytotoxic dose [CD_{50}] per milligram of bacterial protein) was calculated for each *Salmonella* strain from the linear regression of the percentage of HeLa cell survival versus the logarithm of the toxin dilution (3). In each experiment, control cytotoxins (Shiga toxin, SLT-I, and SLT-II) with known cytotoxic activity were also examined to verify the reliability of the assay.

Neutralization studies. Neutralization studies were performed to determine the immunologic relatedness of the cytotoxins produced by *Salmonella* strains to Shiga toxin, SLT-I, and SLT-II. Serial dilutions of *Salmonella* toxins were inoculated into 96-well plates (50 μl per well). Each toxin was then preincubated for 4 h at 37°C with 50 μl of immune serum to purified Shiga toxin (which also neutralized SLT-I), immune serum to SLT-II, and nonimmune serum (control), all in a dilution of 1:50. Preliminary experiments showed that the nonimmune serum neither affected cell survival nor neutralized SLT-I or SLT-II. After the preincubation, HeLa cells were added and the assay was completed as described above; neutralization of cytotoxicity was determined by an increase in HeLa cell survival compared with survival in the control. Purified Shiga toxin and SLT-II were used as positive controls in each experiment; they were incubated with the corresponding immune serum to verify the neutralizing effect of the serum.

DNA hybridization assays. To determine genetic relatedness, *Salmonella* isolates were examined for ability to hybridize with SLT-I and SLT-II DNA probes provided by J. W. Newland and R. J. Neill (Walter Reed Army Institute of Research, Washington, D.C.). The SLT-I probe was a 1,142-base-pair *Bam*HI fragment of the recombinant plasmid pJN37-19, cloned from *E. coli* bacteriophage 933J (25). The SLT-II probe was an 842-base-pair *Sma*I-*Pst*I fragment from the recombinant plasmid pNN110-18, cloned from *E. coli* bacteriophage 933w (26). Since Shiga toxin and SLT-I differ in only three nucleic acids, the SLT-I probe hybridized with Shiga toxin-producing organisms. The probes were extracted from low-temperature-gelling agarose and labeled by nick translation with α-^{32}P-labeled 5′-dCTP (Dupont, NEN Research Products, Boston, Mass.). Colonies of each *Salmonella* strain were transferred from MacConkey agar plates to 541 filter papers (Whatman, Inc., Clifton, N.J.) and lysed as described elsewhere (23). The following control strains were placed on each filter: *Shigella dysenteriae* 1 strain 60R

(produces Shiga toxin), *E. coli* O26:H11 strain H30 (produces SLT-I), *E. coli* C600 (933w) (produces SLT-II), and *E. coli* C600 (negative control). Hybridization was performed under stringent conditions (50% formamide, 42°C, 2× SSC [SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 18 h); filters were then washed four times for 30 min with 2× SSC at room temperature, dried, and exposed to X-ray films.

Characterization of cytotoxic activity. To further define the cytotoxin, one strain of each species (*S. typhi* HD-4580, *S. enteritidis* serotype *heidelberg* strain HD-4214, and *S. choleraesuis* serotype kunzendorf strain BE2-1641) received the following treatments. (i) For heat treatment, 500-μl fractions of the toxins were heated for 30 min to 50, 80, or 100°C. Cytotoxicity was then determined as described above and compared with that of an unheated control. (ii) For trypsin treatment, 250-μl fractions of the toxins were incubated for 30 min at 37°C with trypsin (final concentration, 2 mg/ml; Sigma Chemical Co., St. Louis, Mo.). Soybean trypsin inhibitor (1.0 mg/1.5 mg of trypsin; Sigma) was added to stop trypsin activity. Cytotoxicity was then determined and compared with that of controls incubated with the same volume of medium or with trypsin that had been previously incubated for 30 min at 37°C with trypsin inhibitor. (iii) Molecular weight ranges were determined by ultrafiltration with Amicon membranes (Amicon Corp., Danvers, Mass.). The following fractions of defined molecular weight ranges were prepared: <10,000, 10,000 to 30,000, 30,000 to 100,000, and >100,000. Each fraction was examined for cytotoxicity as described above. Gel filtration chromatography was performed with Sephacryl S-200 (Pharmacia LKB Biotechnology, Piscataway, N.J.) in a column measuring 20 cm by 2 cm^2; 1-ml fractions were collected in 10 mM phosphate buffer with 50 mM NaCl and examined for cytotoxicity. Known molecular weight standards included alcohol dehydrogenase, bovine serum albumin, chemotrypsin, and blue dextran.

Statistical analysis. Two-factor analysis of variance followed by the Bonferroni *t* test for multiple comparisons was used to examine the statistical significance of the differences among the three species and the various clinical sources within each species.

RESULTS

Cytotoxicity assays. Figure 1 shows the amounts of toxin (CD_{50} per milligram of bacterial protein) produced by all *Salmonella* strains examined. All strains showed some cytotoxin production, with amounts ranging from 10 to 1,000 CD_{50}/mg. The geometric means (± standard deviation) of cytotoxin (CD_{50} per milligram of bacterial protein) produced by the three species were 27 ± 2 for *S. typhi*, 65 ± 2 for *S. enteritidis*, and 117 ± 2 for *S. choleraesuis*. Analysis of variance showed that the differences between the species were significant ($P < 0.001$), even though overlap between groups was seen. The range of the amount of toxin produced was highest in *S. enteritidis* species, perhaps a reflection of the heterogeneity of the several serotypes included in this species. However, no serotype that produced significantly different amounts of toxin could be identified. Cytotoxin production of *S. typhimurium*, which is frequently invasive, was similar to that of the whole group (geometric mean, 51 CD_{50}/mg of bacterial protein).

Figure 2 shows the relationship between the amount of toxin produced and the source of the strains. Because of the small number of *S. enteritidis* isolates from blood and cerebrospinal fluid, these strains were grouped together as extraintestinal isolates, representing invasive disease. No significant differences were found between stool isolates and invasive strains for any of the species. Within invasive strains of *S. enteritidis*, there were no significant differences between blood and cerebrospinal fluid isolates. Under the same experimental conditions, *Shigella dysenteriae* 1 and *E. coli* H30 produced about 1,000 times more Shiga toxin and SLT-I, respectively (10^5 CD_{50}/mg), and *E. coli* C600 (933w) produced 10 to 100 times more (10^3 to 10^4 CD_{50}/mg) SLT-II.

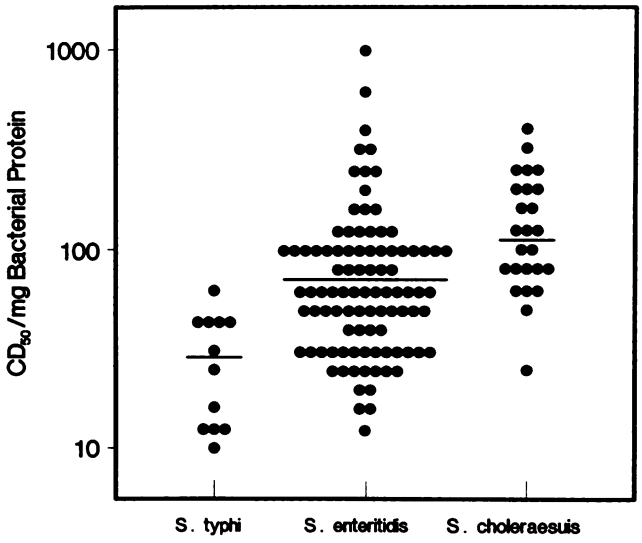

FIG. 1. Amounts of cytotoxin produced by 131 *Salmonella* strains as determined by a [^{3}H]thymidine-labeled HeLa cell assay. Bars represent geometric means. Differences between the species were significant by analysis of variance ($P < 0.001$).

Neutralization studies. Figure 3 shows the results of a

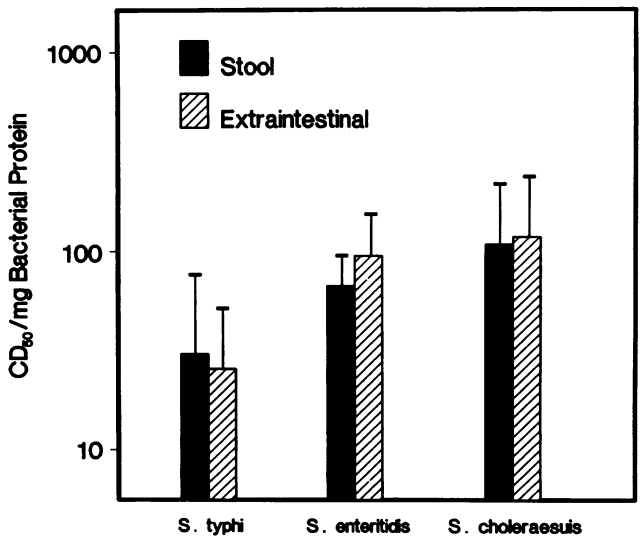

FIG. 2. Relationship between the source of each *Salmonella* isolate and the amount of cytotoxin produced. *S. enteritidis* isolates from blood and cerebrospinal fluid were grouped together as extraintestinal isolates. Results are presented as geometric mean ± standard deviation of each group. No significant differences were found between stool isolates and invasive strains of the same species.

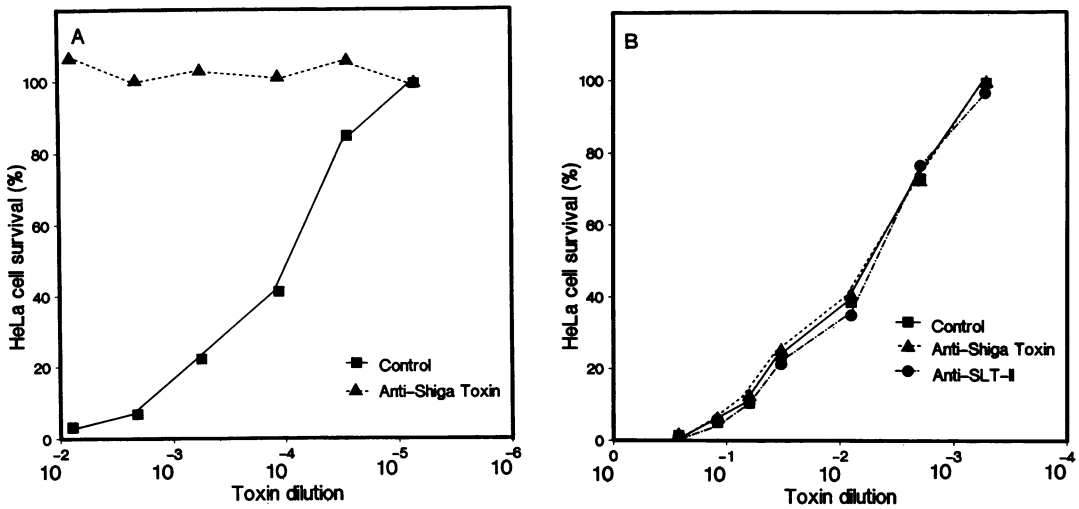

FIG. 3. Neutralization of cytotoxic activity by specific antisera. Cytotoxins were preincubated for 4 h at 37°C with rabbit nonimmune serum (control), rabbit antiserum to purified Shiga toxin (which also neutralizes SLT-I), and rabbit antiserum to SLT-II. Radiolabeled HeLa cells were then added, and cytotoxicity was determined. (A) Typical positive control assay of Shiga toxin. Neutralization of cytotoxic activity was manifested by increased cell survival in each of the toxin dilutions. (B) Typical *Salmonella* strain, for which no neutralization was demonstrated by antiserum to Shiga toxin or SLT-II.

typical neutralization assay. The positive control assay (Fig. 3A) shows neutralization of Shiga toxin by antiserum to purified Shiga toxin. In contrast, antiserum against Shiga toxin or SLT-II failed to neutralize *Salmonella* cytotoxin (Fig. 3B). None of the *Salmonella* strains showed neutralization of cytotoxic activity by these antisera.

DNA hybridization assays. None of the 131 *Salmonella* strains hybridized with DNA probes for SLT-I and SLT-II, whereas *Shigella dysenteriae* 1 and *E. coli* H30 hybridized with SLT-I probe, *E. coli* C600 (933w) hybridized with SLT-II probe, and *E. coli* C600 hybridized with neither probe.

Characterization of cytotoxic activity. Heating of cell-free *Salmonella* cytotoxins to 50°C for 30 min had a minimal effect on cytotoxicity, whereas heating to 80°C destroyed about 80% of the cytotoxicity and heating to 100°C inactivated most of the toxic activity (Fig. 4). Treatment with trypsin destroyed about 80% of the cytotoxic activity of the three *Salmonella* strains, an effect which was specifically blocked by preincubation with soybean trypsin inhibitor (Fig. 5). Trypsin inhibitor itself did not have a significant effect on cytotoxicity. The fractions of defined molecular weight ranges were examined for cytotoxicity. It was found that the fraction in the molecular weight range of 30,000 to 100,000 produced the cytotoxic activity in each of the three *Salmonella* species. Gel filtration chromatography showed that the strains of *S. typhi*, *S. enteritidis*, and *S. choleraesuis* had cytotoxic activity in the fractions with molecular weights of approximately 56,000, 70,000, and 78,000, respectively.

DISCUSSION

All 131 *Salmonella* strains examined produced some degree of cytotoxicity in a quantitative HeLa cell assay. The amounts produced were always lower than the amounts produced by *Shigella dysenteriae* 1 and *E. coli* O157:H7 but similar to those produced by most *Shigella* strains under the same experimental conditions (3). Significant differences were found in the amounts produced by the three *Salmonella* species; *S. typhi* produced the lowest amounts of toxin. Previous studies examined the cytotoxicity of *S. enteritidis* and two strains of *S. typhi* (2, 18, 20, 29) by morphologic criteria. Quantitative comparison of toxin production by different species and sources and comparison with the amounts produced by well-characterized toxin-producing strains were not performed. The radiolabeled assay enabled quantitation of toxin production and these comparisons.

The nature of the toxin(s) produced was partially defined. The cytotoxins produced by all strains examined in this study were genetically and immunologically distinct from Shiga toxin and from SLT-I and SLT-II produced by *E. coli*. Cytotoxic activity was heat labile and sensitive to trypsin treatment, indicating that the active component is probably protein in nature. It is not clear whether one toxin or more

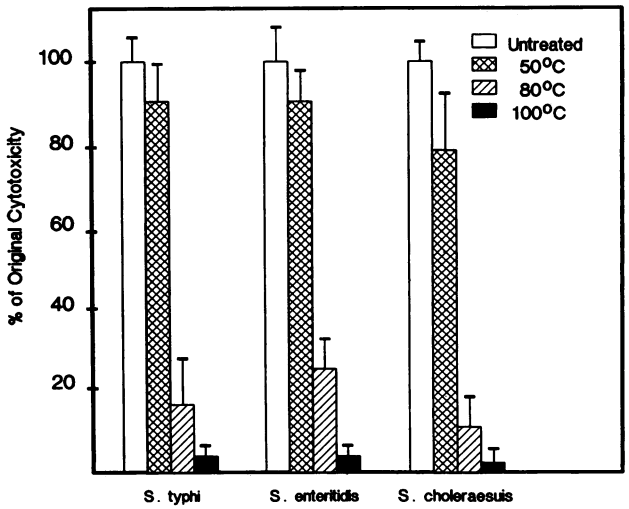

FIG. 4. Effect of heat treatment (30 min each) on cytotoxic activity. Results are expressed as percentage of cytotoxicity of an untreated control and presented for each group as mean ± standard deviation of six determinations.

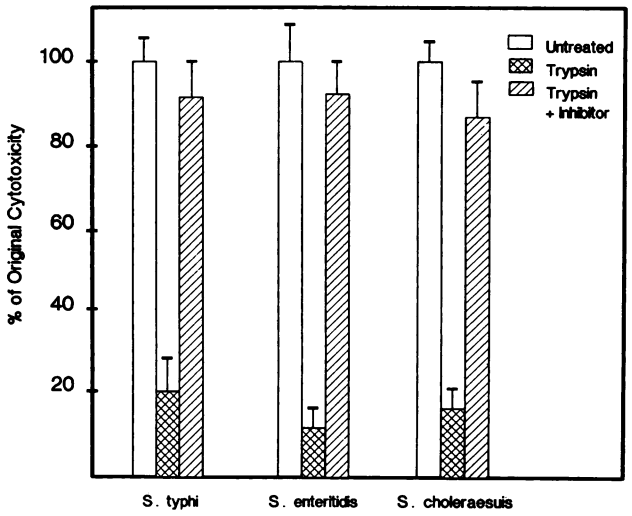

FIG. 5. Effect of trypsin treatment on cytotoxic activity. Toxins were incubated at 37°C for 30 min with trypsin (final concentration, 2 mg/ml) and with trypsin that had been previously preincubated with trypsin inhibitor (1.0 mg of inhibitor per 1.5 mg of trypsin). Results are expressed as percentage of cytotoxicity of an untreated control and presented for each group as mean ± standard deviation of six determinations.

are involved. Previous studies have shown that four *S. enteritidis* strains produce heat-labile cytotoxic activity, with inhibition of protein synthesis in Vero cells in vitro (20). Further studies by Koo et al. have shown decreased protein synthesis in rabbit intestinal epithelial cells both after infection of intestinal loops with *S. enteritidis* strains and after exposure of the epithelial cells to cell-free lysates of these strains in vitro (21).

The role of cytotoxins in the pathogenesis of salmonellosis has not been clarified. It is well established that the species of *Salmonella* is important in determining the clinical and pathological manifestations in both natural infection in humans and experimental disease in animals (1, 11, 32, 34). The fact that significant differences were found in the amounts of toxin produced by *Salmonella* species suggest that cytotoxin production may play a role in pathogenesis. *S. typhi* produced the lowest amount of toxin, an amount similar to that produced by nonpathogenic enteric *E. coli* (8) *S. enteritidis*, which causes more enteric symptoms and inflammatory diarrhea than does *S. typhi* (9, 34), produced higher levels of cytotoxin. This observation suggests that cytotoxin may play a role in the local damage to intestinal mucosa that results in enteric symptoms and inflammatory diarrhea and is consistent with previous findings suggesting that cytotoxin production in shigellosis is related to enteric findings, such as fecal leukocytes and occult blood in stool (31). The pathogenesis and invasiveness of *S. typhi* may be related to other noncytotoxic factors such as envelope virulence antigen (Vi). It is not clear from this study whether cytotoxin production plays a role in invasiveness or bacteremia, since no significant differences were found between enteric and invasive isolates of the same species. A recent study by Reitmeyer et al. showed that *S. enteritidis* cytotoxin was a component of bacterial outer membrane (33). This observation is in contrast to findings with other cytotoxins of enteric pathogens, which are usually soluble factors released by the bacteria. It has been suggested that this location enables direct contact of the *Salmonella* toxin with host cells, causing damage and perhaps promoting invasion. Thus, relatively low amounts of toxin may have a role in virulence.

The precise role of *Salmonella* cytotoxin in the pathogenesis of diarrhea and invasiveness requires additional study. This research will require further purification of the toxin, clarification of the relationship with *Salmonella* enterotoxin, and examination in experimental models.

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LITERATURE CITED

1. **Ascher, D. P.** 1987. *Salmonella choleraesuis* infection in childhood. Mil. Med. **152**:376–377.
2. **Baloda, S. B., A. Faris, K. Krovacek, and T. Wadstrom.** 1983. Cytotoxic enterotoxins and cytotoxic factors produced by *S. enteritidis* and *S. typhimurium*. Toxicon **21**:785–790.
3. **Bartlett, A. V., III, D. Prado, T. G. Cleary, and L. K. Pickering.** 1986. Production of Shiga toxin and other cytotoxins by serogroups of Shigella. J. Infect. Dis. **154**:996–1002.
4. **Barlett, T. G.** 1979. Antibiotic-associated pseudomembranous colitis. Rev. Infect. Dis. **1**:530–539.
5. **Centers for Disease Control.** 1984. Human *Salmonella* isolates—United States, 1983. Morbid. Mortal. Weekly Rep. **33**:693–695.
6. **Centers for Disease Control.** 1987. Summaries of notifiable diseases in the United States, 1986. Morbid. Mortal. Weekly Rep. **35**:1–57.
7. **Chalker, R. B., and M. J. Blazer.** 1988. A review of human salmonellosis. III. Magnitude of *Salmonella* infection in the United States. Rev. Infect. Dis. **10**:111–124.
8. **Cleary, T. G., J. J. Mathewson, E. Faris, and L. K. Pickering.** 1985. Shiga-like cytotoxin production by enteropathogenic *Escherichia coli* serogroups. Infect. Immun. **47**:335–337.
9. **Clyde, W. A., Jr.** 1957. Salmonellosis in infants and children. A study of 100 cases. Pediatrics **17**:175–182.
10. **Cohen, J. I., J. A. Bartlett, and G. R. Corey.** 1987. Extra-intestinal manifestations of *Salmonella* infections. Medicine **66**: 349–388.
11. **Edelman, R., and M. M. Levine.** 1986. Summary of international workshop on typhoid fever. Rev. Infect. Dis. **8**:329–349.
12. **Finkelstein, R. A., B. A. Marchlewicz, R. J. McDonald, and M. Boesman-Finkelstein.** 1983. Isolation and characterization of cholera-related enterotoxin from *S. typhimurium*. FEMS Microbiol. Lett. **17**:239–241.
13. **Gianella, R. A., S. B. Formal, G. J. Dammin, and H. Collins.** 1973. Pathogenesis of salmonellosis. Studies of fluid secretion, mucosal invasion and morphologic reaction in the rabbit ileum. J. Clin. Invest. **52**:441–453.
14. **Gransden, W. R., M. A. S. Dam, J. D. Anderson, J. E. Carter, and H. Lior.** 1986. Further evidence associating hemolytic uremic syndrome with infection by verotoxin producing *E. coli* O157:H7. J. Infect. Dis. **154**:522–524.
15. **Guerrant, R. L., C. A. Wanke, and R. A. Pennie.** 1987. Production of unique cytotoxin by *Campylobacter jejuni*. Infect. Immun. **55**:2526–2530.
16. **Jiwa, S. F. H.** 1981. Probing for enterotoxigenicity among the salmonellae: an evaluation of biological assays. J. Clin. Microbiol. **14**:463–472.
17. **Karmali, M. A., M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior.** 1985. An association between idiopathic hemolytic uremic syndrome and infection by verotoxin producing *Escherichia coli*. J. Infect. Dis. **151**:775–782.
18. **Ketyi, I., S. Pacsa, L. Emody, A. Vertenyi, B. Kocsis, and B. Kuch.** 1979. *Shigella dysenteriae* 1-like cytotoxic enterotoxins produced by *Salmonella* strains. Acta Microbiol. Acad. Sci.

Hung. **26**:217–223.
19. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18**: 775–779.
20. **Koo, F. C. W., and J. W. Peterson.** 1983. Cell-free extracts of Salmonella inhibit protein synthesis and cause cytotoxicity in eukaryotic cells. Toxicon **21**:309–320.
21. **Koo, F. W., J. W. Peterson, C. W. Houston, and N. C. Molina.** 1984. Pathogenesis of experimental salmonellosis: inhibition of protein synthesis by cytotoxin. Infect. Immun. **43**:93–100.
22. **Kuhn, H., H. Tschape, and H. Rische.** 1978. Enterotoxigenicity among salmonellae—a prospective analysis for a surveillance programme. Zentralbl. Bakteriol. Hyg. 1 Abt. Orig. **240**:171–183.
23. **Maas, R.** 1983. An improved colony hybridization method with significantly increased sensitivity for detection of single genes. Plasmid **10**:296–298.
24. **Marques, L. R. M., M. A. Moore, J. G. Wells, I. K. Wachsmuth, and A. D. O'Brien.** 1986. Production of Shiga-like toxin by *Escherichia coli*. J. Infect. Dis. **154**:338–341.
25. **Newland, J. W., N. A. Strockbine, S. F. Miller, A. D. O'Brien, and R. K. Holmes.** 1985. Cloning of Shiga-like toxin structural genes from a toxin converting phage of *Escherichia coli*. Science **230**:179–181.
26. **Newland, J. W., N. A. Strockbine, and R. J. Neill.** 1987. Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II. Infect. Immun. **55**:2675–2680.
27. **Niesel, D. W., C. E. Chambers, and S. L. Stockman.** 1985. Quantitation of HeLa cell monolayer invasion by *Shigella* and *Salmonella* species. J. Clin. Microbiol. **22**:897–902.
28. **O'Brien, A. D., and R. K. Holmes.** 1987. Shiga and Shiga-like toxins. Microbiol. Rev. **51**:206–220.
29. **O'Brien, A. D., G. D. LaVeck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **146**:763–769.
30. **Peterson, J. W., N. C. Molina, and C. W. Houston, and R. C. Fader.** 1983. Elevated cAMP in intestinal epithelial cells during experimental cholera and salmonellosis. Toxicon **21**:761–765.
31. **Prado, D., T. G. Cleary, L. K. Pickering, C. D. Ericsson, A.V. Bartlett, III, H. L. DuPont, and P. C. Johnson.** 1986. The relation between production of cytotoxin and clinical features in shigellosis. J. Infect. Dis. **154**:149–155.
32. **Reed, W. M., H. J. Olander, and H. L. Thacker.** 1986. Studies of the pathogenesis of *Salmonella typhimurium* and *Salmonella choleraesuis* var kunzendorf infection in weaning pigs. Am. J. Vet. Res. **47**:75–83.
33. **Reitmeyer, J. C., J. W. Peterson, and K. J. Wilson.** 1986. *Salmonella* cytotoxin: a component of bacterial outer membrane. Microb. Pathog. **1**:503–510.
34. **Rennels, M. B., and M. M. Levine.** 1986. Classical bacterial diarrhea: perspectives and update—*Salmonella, Shigella, Escherichia coli, Aeromonas* and *Plesiomonas*. Pediatr. Infect. Dis. **5**:S91–S100.
35. **Sandefur, P. D., and J. W. Peterson.** 1977. Neutralization of *Salmonella* toxin-induced elongation of Chinese hamster ovary cells by cholera antitoxin. Infect. Immun. **15**:988–992.
36. **Takeuchi, A.** 1967. Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. **50**:109–136.
37. **Wallis, T. S., W. G. Starkey, J. Stephen, S. J. Haddon, M. P. Osborne, and D. C. A. Candy.** 1986. Enterotoxin production by *S. typhimurium* strains of different virulence. J. Med. Microbiol. **21**:19–23.