THE INFLUX OF FLUID INTO INTESTINES OF SUCKLING MICE EXPOSED TO HUMAN AND EQUINE ISOLATES OF *SALMONELLA*

By

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ABSTRACT

THE INFLUX OF FLUID INTO INTESTINES OF SUCKLING MICE EXPOSED TO HUMAN AND EQUINE ISOLATES OF *SALMONELLA*

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Human and equine isolates of *Salmonella* were examined for enterotoxin-like activity in the suckling mouse assay. Culture supernatant fluids of several isolates induced influx of fluid in suckling mice. The activity depends on the presence of organisms; filtrates, concentrated filtrates and vigorously centrifuged culture preparations did not evoke fluid accumulation in mice. Negative results observed with heat-killed and sonicated organisms suggest viability is a requirement. Results of lethality studies in adult mice suggest suckling mice positive isolates are more virulent for adult mice than are negative strains. A high percentage of suckling mice positive isolates exhibited mannose sensitive hemagglutinating activity of guinea pig cells while none of the suckling mice negative strains exhibited this activity suggesting a correlation between the presence of pili and suckling mice activity. Enterotoxin-like activity does not appear to depend on multiplication of *Salmonella* within the suckling mouse gut.

DEDICATION

To my family

Parents, Clarence P. and Genevieve C. Simon;

Brother, Gene A. Simon;

and

Sisters, Carol A. Nurenberg and Ellen M. Simon.

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INTRODUCTION

Salmonella gastroenteritis is one of the most common zoonotic communicable diseases of man and animals. In 1978, 29,410 human cases were reported in the United States (19). Further, Morse et al. estimated that at least 10% of the U.S. equine population is or has been infected with *Salmonella* (119).

Relatively little is known on a cellular or molecular level regarding the events occurring from when *Salmonella* gain entry to the intestinal tract until diarrheal symptoms are observed. *Salmonella* strains show marked variation in their ability to penetrate tissues and cause disease. Giannella et al. have shown that only invasive strains produce diarrhea, but mucosal invasion alone is not sufficient to account for the fluid response in experimental infection (63). To identify the property of the organism that is responsible for fluid loss, many investigators have utilized the same tools to examine *Salmonella* that were important in the characterization of *V. cholerae* and *E. coli* enterotoxins (63, 103, 104, 146, 147, 151). Since *Salmonella* do react positively in some of the enterotoxin models it was hypothesized that *Salmonella* produce a toxin that is responsible for the diarrhea observed in Salmonella

gastroenteritis. The initial purposes of this study were to examine clinical isolates of *Salmonella* from humans and horses for enterotoxin activity in the suckling mouse assay and subsequently to characterize the toxin. When preliminary investigations suggested that the activity of *Salmonella* in the suckling mouse might not be due to an exotoxin, attempts were made to define the nature of the suckling mouse response and to evaluate the use of this model as a monitor for the pathogenicity of *Salmonella* strains *in vivo*.

LITERATURE REVIEW

Bacterial diarrhea is a significant cause of world-wide morbidity and mortality. At least two major pathogenic mechanisms of bacterial diarrhea are recognized, the so-called toxigenic diarrhea, and the invasive diarrhea. In the toxigenic diarrhea an enterotoxin produced by organisms in the small intestine evokes active fluid secretion by the intestinal epithelium. Prototypes for this mechanism are Vibrio cholerae and non-invasive Escherichia coli. Invasive organisms such as Shigella and Salmonella penetrate the intestinal mucosa and multiply within the mucosa causing damage to the tissue and fluid loss (135, 164). While the mechanism of fluid loss in the toxigenic diarrheas has been well characterized, little is known of the exsorptive mechanism in the invasive diarrheas.

Pathophysiology of Enterotoxigenic Organisms

Cholera is characterized by massive fluid loss leading to severe dehydration and shock (132). Certain strains of E. coli cause a similar clinical condition in man (36, 105, 109, 139, 153) and animals (87, 113, 157). In the past, diarrhea producing strains of E. coli belonged to certain serotypes and for this reason attempts were made

to associate enterotoxigenicity with particular somatic antigens (76, 79, 144). While these studies failed to find a correlation, a relationship was found between distinct capsular (K) antigens and toxigenicity (87, 91, 92, 113, 115). It was observed that enterotoxigenic E. coli (ETEC) isolated from diseased swine frequently possess the K88 surface antigen (91, 92, 113, 114, 115) and K99 antigen has been associated with ETEC cultured from calves and sheep (87).

Two steps are necessary for pathogenesis of these enterotoxigenic organisms. First, the organisms must adhere to the intestinal mucosa, and second, a toxin must be produced and released. Numerous investigators have studied surface properties of organisms that might be responsible for the adherence. In V. cholera, adhesion has been associated with motility (54, 91) and although the exact nature of this antigen is not known, the adhesiveness may be associated with the bacterial flagellum (54, 90). Non-motile vibrios are unable to adhere and attached vibrios show little movement after adherence (90). In ETEC, adherence is determined by the presence of certain K antigens in animals and colonization factor antigens in humans. These antigens are plasmid mediated, non-flagellar, surface appendages composed of protein believed to be pili (114). In piglets ETEC strains possessing K88 antigen are

able to adhere, colonize, and produce disease in the small intestine, and if the antigen is lost, these strains no longer have these abilities even though they retain their enterotoxigenicity (91, 114). Evans et al. demonstrated that a human strain of ETEC possessed a surface associated colonization factor (CF) and caused diarrhea in infant rabbits. A laboratory passed derivative of the strain lacking the pilus-like surface structures failed to colonize the young rabbits or induce diarrhea. Cell-free culture supernatant fluids produced the same enterotoxic responses in the rabbit (44). Furthermore, passive protection of infant rabbits with anti-CF serum was demonstrated (44). Morgan et al. showed vaccination of pregnant gilts with purified pili protects the piglets against diarrhea caused by ETEC strains possessing the same pili, but does not protect from enterotoxigenic strains possessing different pili (91). Thus, it has been concluded that certain pili may play an important role in ETEC diarrhea.

Toxin production in V. cholera is coded for by a chromosomal gene (166). Enterotoxins of E. coli are coded for by plasmids often in association with adherence antigens and drug-resistance factors (80, 81, 155, 156).

V. cholera enterotoxin (CT) has been isolated (53) and found to have a molecular weight of 84,000 daltons. It is composed of two major subunits, A and B. The B

subunit (choleragenoid), the cholera toxoid, is immunogenic. It is the component that binds to intestinal membranes, but lacks toxin activity. Subunit A is responsible for toxin activity but is not antigenic (for a recent review refer to Richards and Douglas, 137).

ETEC strains produce two different types of enterotoxins with unique biochemical and physical properties. The two toxins have been designated ST and LT based on thermostability. ST is stable when heated at 65°C, with only a small decrease in toxic activity after heating at 100°C for 30 minutes (58). Recently purified by Alderete and Robertson (2), ST was determined by sodium dodecyl sulfate gel electrophoresis and gel chromatography to have a molecular weight of 4,400 daltons. ST activity is resistant to acid, trypsin, and pronase (2, 88). In contrast, LT activity is destroyed by heat at 65°C, and is acid labile (33). Molecular weight estimated by gel chromatography was 102,000 daltons (33), however a smaller molecular weight of 20,000 was obtained when an ETEC strain was polymixin treated for rapid release of LT. This polymixin released LT has the same biological activities as the naturally released LT (48).

The CT receptor site is located in the glycocalyx, Gm_1 ganglioside has been suggested as the natural receptor (85). LT also binds to this ganglioside but with lower

affinity (21, 74, 85, 100). Treatment of rabbit intestine with choleragenoid prior to administration of LT does not significantly alter binding of LT (21, 74, 85). This suggests possibly different binding sites or different affinities for the toxins. There is little information about ST binding properties although there is evidence that binding site of ST is different from the glycocalyx Gm_1 ganglioside receptor described for CT (21).

Both LT and CT have been shown to activate membrane bound adenylate cyclase which converts adenosine 5'-triphosphate (ATP) to cyclic AMP (cAMP) (68, 69, 75). Reaction requirements for each, as demonstrated by Gill (68, 69), are ATP, nicotinamide adenine dinucleotide, and an undefined soluble protein. Elevated levels of cAMP result in a net secretion of chloride and inhibition of sodium absorption with loss of water. ST does not appear to raise cAMP levels while it does significantly alter cGMP levels. Administration of cGMP alone will evoke a fluid response similar to ST (62, 125). Evidence suggests that activation of cGMP is intimately involved in the mechanism of fluid loss in ST diarrhea.

In addition to the similarities in biological properties of CT and LT there also appears to be an antigenic relatedness. Anti-cholera toxin will neutralize toxic activity of CT and LT <u>in vivo</u> (20, 33, 100) as well

as showing immunologic relationships in immunodiffusion (20, 33, 48). Similarly, anti-LT sera neutralizes and produces a precipitin band with both LT and CT (20, 33, 48, 155). Klipstein et al. reported weak neutralization of ST with anti-LT and anti-CT (100) in contrast to Smith and Gyles who found no neutralization of ST with anti-LT (155).

Animal and Cell Models for Detection of Enterotoxin Production

A large number of animal and cell models have been utilized to study enterotoxin activity. The rabbit ileal loop was one of the first systems used (41, 63, 85, 151, 164), but since it is a rather cumbersome procedure many attempts have been made to find more reliable methods. Some of these methods are rabbit skin permeability (13, 23, 40, 47, 104, 146), suckling mouse (9, 26, 58, 103), infant rabbit (44), infant pig (114, 115), various tissue culture assays such as Y-1 adrenal cell (30, 78, 143), Chinese hamster ovary cell (74, 75, 147), Vero cell (102, 159). Attempts to develop a truly *in vitro* assay have included passive immune hemolysis (16, 45), ELISA (160), and RIA (131). Enterotoxic activity in these models has been described for several organisms *V. cholera* (9, 13, 23, 25, 75), ETEC (26, 30, 40, 41, 47, 58, 143), *Salmonella* (63, 103, 146, 147, 164), *Yersinia enterocolitica* (128,

129), Klebsiella pneumoniae (98, 99, 101), and Enterobacter cloacae (101). Due to an imperfect understanding of the exact mechanism involved in each of the systems, correlations are sometimes difficult to make. The enterotoxic factor reacting in one model may not be the same factor reacting in another model (13, 102). A given system may respond to more than one enterotoxic factor and thus enterotoxic factors of different genera reacting in the same model are not necessarily identical (9, 41, 103, 151, 152). Looking at the parameters of a few of the assays may clarify interpretation somewhat.

The rabbit ileal loop was used in the early work on CT (25). In this procedure toxin material is injected into ligated sections of adult rabbit ileum. After an appropriate incubation time, usually 18 hours, the ileal sections are excised and measurements are taken of the volume of fluid accumulated within the segment and the length of the segment. LT of ETEC reacts similarly to CT in this model, fluid accumulation is maximal at greater than 10 hours, response is sustained for several hours. ST also evokes a fluid response in this model but is maximal within 4-6 hours, resolves rapidly after 6 hours, and net secretion is less than with LT (41). Some Salmonellae elicit a positive response in this model (63, 151, 164). Giannella et al. used this model to study both

invasive and noninvasive strains of S. typhimurium. The noninvasive strains did not evoke a fluid response while three out of six invasive strains elicited a positive response. This suggests invasion plus another factor both are involved in the diarrheal response. Fluid accumulation could be demonstrated only in the presence of whole organisms; sterile filtrates and lysates of the bacteria yielded no response. This is in contrast to the cell-free toxin activity of LT, ST, and CT. Giannella attempted to correlate his rabbit loop data with mouse and guinea pig LD_{50}'s and diarrhea in rhesus monkeys, but results were inconclusive. Sakazaki et al. report better activity with cell-free filtrates of Salmonella than with whole organism preparations (145). They obtained positive ileal loop responses in 4 of 13 strains of Salmonella using live cultures and 11 of the 13 were positive using culture filtrates. The data did exhibit considerable variability, 6 of the strains reacting positively in only 50% of the loops tested. Interpretation of the data is difficult since responses were merely listed as positive or negative. Expression of the data as a mean of the actual fluid accumulated in each test would have been more meaningful. Sedlock and Deibel developed an improved ileal loop procedure by washing the intestine with a mucus lysing agent, N-acetyl-L-cysteine, prior to injection of toxin preparation

(151, 152). Using this technique they were able to consistently demonstrate fluid accumulation with cell-free filtrates of Salmonella cultures. It was suggested that removal of the mucus removed a component which may inhibit the enterotoxin or cover a receptor site of the toxin. Invasive Salmonella are thought to eliminate the intestinal mucus, in an unknown manner, as evidenced by the observed loss of crypt mucus and villus goblet cell mucus associated with a Salmonella invasion (63, 95, 140). This could allow a possible toxin access to its site of action.

The rabbit skin permeability assay was another system developed which detects enterotoxin activity of V. cholera (23), ETEC (40, 47), and Salmonella (146). Culture filtrates of these organisms produce erythema, induration, and increased capillary permeability when injected into the skin of rabbits. Reading of the test is enhanced by injection of a protein-binding dye (Evan's blue) several hours after the culture filtrates. A rapid blanching reaction may be observed if the dye is injected 1 hour after the filtrates. This can be demonstrated with E. coli and Salmonella and is caused by a factor which is heat stable, in contrast to the delayed permeability factor which is heat labile (146). The possibility that the blanching factor contains LPS has not been excluded. Peterson was able to detect the delayed permeability

factor of Salmonella only after chromatography of culture filtrates of Sephadex G100 which appears to remove some inhibitor-like substance (146). Kuhn et al. observed delayed permeability factor activity on directly assayed culture supernatant fluids of Salmonella (104).

The suckling mouse model detects enterotoxin activity of E. coli ST (26, 158), CT (9) and Salmonella (103). It was developed by Dean et al. to detect enterotoxin production by E. coli (26). He used mice 1-4 days old, injected the test material through the body wall directly into their milk-filled stomachs. The mice were sacrificed at 4 hours, the intestines removed and examined for fluid accumulation. Activity, expressed as gut to remaining body weight ratio, was heat stable. Selected parameters of the assay were modified by Giannella (58), who suggested that incubation time be changed to 3 hours, bacterial growth medium be casamino acids-yeast extract broth, and that roller cultures be used to obtain better toxin production than shaker cultures. Heat stability of the toxin was confirmed, but showed a small decrease in activity after heating at 100°C for 30 minutes. The toxic activity was stable to freezing at -20°C for 6 months. The assay was again studied by Moon et al. who reported optimal results using mice less than 6 days old, held at room temperature (112). Older mice and mice held at 37°C tended

to respond with diarrhea rather than fluid accumulation, thus giving falsely negative results. A comparative evaluation of several strains of inbred mice and Swiss mice concerning their susceptibility to ST revealed that all strains responded to the toxin, but that the Swiss mice gave the most consistent results (130). Koupal and Deibel described the assay for evaluation of Salmonella (103). Activity was demonstrated using BHI culture supernatants as well as washed organisms. The optimal incubation period was 2.5 hours. Heating above 70°C destroyed the toxic activity, freezing at -20°C resulted in a slight decrease in activity. These data suggested that the toxin activity was associated with the outer membrane portion of the organism, but did not involve LPS. Later Sedlock, Koupal, and Deibel partially purified an enterotoxin factor which demonstrated activity in the rabbit ileal loop assay (152). They presumed this factor to be identical to the one reported in the suckling mouse, however no comparative studies were made. In addition to changing animal models these investigators examined cell-free culture filtrates and ultrafiltration concentrates instead of culture supernatants and cell extracts used in the suckling mouse model. Even though it is not certain that this activity is identical to that described previously, they were able to achieve a fifty-fold purification on a DEAE column, found the factor was

heterogeneous in size eluting in a broad band on Sephadex G100 chromatography, also a small amount of enterotoxic activity passed through a PM30 membrane while most of the activity was retained.

In vitro cell systems provide simple, sensitive tools in the study of enterotoxins. The Y-1 adrenal cell (30, 31, 143) and Chinese hamster ovary cell (CHO) (102, 159) are used most frequently. In the Y-1 assay LT and CT induce morphological rounding and steroidogenesis in monolayer cultures. One strain of S. typhimurium tested in this system proved negative (30). The CHO assay responds by activation of adenylate cyclase by LT and CT with accumulation of cAMP and cell elongation. Anti-serum to CT inhibits enterotoxic effects of both CT and LT. The sensitivity of this test for CT is 100 to 10,000 times greater than the rabbit ileal loop and rabbit skin permeability tests. It is 5-100 times more sensitive to LT than is the rabbit loop and the skin permeability factor (75). Peterson described reactivity of Salmonella in the CHO cell assay (147). Activity could only be demonstrated on chromatographed culture filtrates similar to the phenomenon described for the permeability factor assay. This activity is neutralizable with antisera to CT.

Salmonellosis in Horses

Equine salmonellosis has long been recognized as a serious problem. It has been estimated that 5-10% of the U.S. equine population is or has been infected (119). The young, aged and stressed animals are the most frequently inflicted and the infection in these animals has a very high mortality rate.

Three types of salmonella-infected horses may exist: the horse with diarrhea that is shedding organisms, the carrier with fecal shedding of organisms but without diarrhea, and the carrier without fecal shedding of organisms (119). Long after the horse has recovered from diarrhea, Salmonella may still be recovered from feces of some cases. Morse et al. reported shedding persisting as long as 3½ months (119), and Bryans et al. reported positive fecal cultures at 6-14 months (18), at which time the monitoring was discontinued. This carrier state poses problems such as deciding when the horse may safely return to the herd. There may be equine carriers that do not shed Salmonella until stressed. A similar situation has been reported in swine stressed by a 3½ hour truck ride (171). Confinement of horses that have been stressed by illness and transportation in veterinary clinics provides the ideal situation for initiating outbreaks. Stress plays a dual role in the pathogenesis of equine salmonellosis, it

initiates shedding of the organism and predisposes the horse to a serious infection. Stress conditions that have been associated with equine salmonellosis are surgery (7, 117, 119, 154), overtraining (117, 119), parasitic worm infestation and anthelmintic medication (7, 27, 117, 168, 170), tetracycline therapy (8, 117, 127), infection such as nephritis, pneumonia, metritis (117) and hot humid weather (119, 171).

The clinical form the disease manifests varies with the age and health of the host. In foals with salmonellosis, 90% will be severely affected with the peracute type of infection which progresses rapidly to septicemia (18, 119, 170). Onset is sudden, fever 40°C or greater is apparent with depression and anorexia. The fetid, profuse, mucous diarrhea is accompanied by severe dehydration and death ensues within 2-3 days. Mares manifest a much milder form of the disease. They are often almost asymptomatic, the only signs being anorexia, soft "cowpie" feces, slight fever, the condition persists about 4-5 days. The animal usually recovers without complication but the infection may progress to the chronic phase. When this occurs temperature is frequently normal, soft diarrhea continues, weight loss and dehydration are marked. This condition may persist for weeks to even months. Generally after a course of 5 weeks the animal becomes so weak it will eventually succumb to the infection.

Colitis "X" is a term used to describe a peracute condition in horses characterized by severe diarrhea and signs of toxemia, of unknown etiology, possibly endotoxemia (138). Clinical signs of colitis X being similar to those of acute salmonellosis has led some authors to believe that some cases of colitis X are really salmonellosis. Salmonella may not be recovered from every fecal sample taken from diseased horses known to be shedding the organism. In light of this difficulty in recovering Salmonella consistently, perhaps repeated fecal cultures should be done on horses with diarrhea before a diagnosis of colitis X is made (127, 154).

The serotypes of Salmonella most commonly isolated in Equidae are S. typhimurium, S. anatum and S. agona (17, 119, 154). Morse et al. reported more severe disease associated with S. anatum than S. typhimurium (119), while Smith et al. found S. typhimurium more often associated with severe disease. S. agona appears to be of low pathogenicity isolated mainly from clinically normal horses (154). Since it belongs to Group B salmonellae as does S. typhimurium it is important to serotype further to distinguish between the two.

The efficacy of antimicrobial therapy in the treatment of equine salmonellosis is doubtful if not discouraging (118, 119). In vitro sensitivity patterns appear to have

little bearing on eliminating salmonellae from the feces. Fluid and electrolyte replacement are essential therapies.

Salmonellosis in Humans

The frequency with which salmonellosis is encountered in man is impressive. It presents a variety of syndromes and it may be associated with a number of other disease etiologies. Despite recent advances of modern man in food processing and medicine, Salmonella has survived and flourished. Salmonellae can withstand the spray-dry processing of eggs (4, 15, 122, 123, 124, 149), boiling of eggs for seventeen minutes at 56°C (158), the pasteurization of milk (29) as well as the processing procedure of non-fat dry milk (56).

Salmonellosis in man is usually classified in four main groups of clinical manifestations: gastroenteritis, typhoid or septic syndrome, focal manifestations and the carrier state (148). One type of disease frequently overlaps or leads to another so that sharp differentiations are not always possible.

Gastroenteritis is by far the most common clinical condition, being responsible for at least 70% of the cases (148). This percentage is probably higher considering the number of minor cases that go without medical attention and diagnosis. The diarrhea ranges in severity from mild irritation to a profuse watery form with rapid dehydration (5,

61, 124, 148). Nausea, abdominal cramps, vomiting, and mild fever are common signs. The aged and infants exhibit the most severe symptoms. The incubation varies from 8-72 hours, most commonly symptoms are observed 20-48 hours after infection (122). The course of the illness is generally 1-4 days. S. typhimurium is by far the most commonly isolated strain in this condition.

Typhoid-like or septic syndrome usually presents as "fever of unknown origin." The fever in most cases reported by Saphra and Winter (148) has a spiking septic character, and was only rarely continuous simulating typhoid fever. The fever and malaise are often the only signs and may last from a few days to several weeks. Black et al. (14) report that in young adults with salmonellosis the presence of lymphadenopathy, splenomegaly, fever and atypical lymphocytes has on several occasions led to erroneous diagnosis of infectious mononucleosis. S. cholerasuis is the most common isolate of septic fever while S. paratyphi A and B and S. panama most frequently present the typhoidal signs which in general are milder than those caused by S. typhi.

Saphra and Winter reported focal manifestations in 7.4% of the cases studied (148). The localized inflammatory processes in some were related to the gastrointestinal tract such as peritonitis, cholecystitis, salpingitis,

appendicitis. Other parts of the body such as lungs, spleen, brain, skin, or heart valves were also found to be involved. Abscesses were common. In most cases diagnosis required surgery and culture of the inflamed tissue. Salmonella has been implicated in pneumonia, osteomyelitis, urinary tract infection, meningitis, and subacute bacterial endocarditis (14, 148). S. cholerasuis is most often responsible for these focal manifestations.

The carrier rate of Salmonella is around 0.2% (149). The carrier state may follow asymptomatic as well as symptomatic cases of salmonella infections. The condition may be transient or the organism may take up permanent residence in the gall bladder, but fortunately this is not a common occurrence. Salmonella carriers are potential health hazards when employed in food handling or hospitals. Many outbreaks have been traced to such people (51, 150). Antibiotic therapy of salmonella carriers is not usually effective in eliminating the infection and may even prolong the presence of the organism (3, 28, 148).

It is worth mentioning that while certain strains of Salmonella are commonly associated with a specific clinical form of salmonellosis, i.e., S. cholerasuis causes most of the septicemias, all Salmonella have the potential to cause any of the syndromes that have been described. It is not even uncommon for a patient to be infected with

more than one strain of Salmonella simultaneously (6, 15, 56). Some double infections probably go unnoticed since most laboratories routinely examine only one suspicious colony for the identification of Salmonella.

Most cases of salmonellosis result from consumption of contaminated food. Since birds are the largest single reservoir of Salmonella, it follows that contaminated poultry, eggs, and egg products are responsible for a large number of epidemics (4, 150). It also follows that the incidence of the illness peaks during the summer months (19) where high temperatures, picnics and poor refrigeration favor the multiplication of the organisms within food. The required infecting doses of different serotypes vary considerably, even strains of a given serotype show as much as a two log variation (122). McCullough et al. reported infective doses in man of S. anatum to be $5.9\text{-}670 \times 10^5$ organisms, S. meleagradis $7.7\text{-}50 \times 10^6$ organisms, S. newport $1.52\text{-}13.5 \times 10^5$ organisms, S. derby 15×10^6 organisms, S. bareilly $1.25\text{-}17 \times 10^5$ organisms and S. pullorum $1.3\text{-}10 \times 10^9$ organisms (122, 123, 124). These studies were done in normal individuals. Infants, the aged, and compromised hosts are likely to have a lower minimal infective dose.

Salmonella is one pathogen that is not likely to be erradicated since it infects such a large population

of animals and exists in extra-host environments (117). This organism will continue to cause its share of morbidity and mortality for years to come.

Pathogenesis of Salmonella Gastroenteritis

Salmonella characteristically have the ability to invade the intestinal mucosa and cause a diarrheal response in the host. The exact mechanism by which the pathogen enters the mucosa and evokes fluid secretion is unknown. Giannella et al. have demonstrated that invasion alone is not enough to cause fluid exsorption. A strain of S. typhimurium SL1027 invades the mucosa but does not evoke a fluid response. Some other invasive strains do evoke a fluid response while noninvasive strains do not cause diarrhea (63). This work was supported and expanded by Sedlock and Deibel who demonstrated that cell-free culture filtrates of an invasive toxigenic Salmonella strain produce a very weak response in the rabbit ileal loop when administered alone, but when tested in the presence of strain SL1027 produced a dramatic response. There appears to be a definite requirement for invasion plus another factor for fluid exsorption to occur (151).

Changes that occur in the gut during salmonella gastroenteritis have been studied in the hope of defining the pathophysiological mechanism behind the diarrheal

response. Morphological changes have been examined in guinea pigs (161, 162), mice (163), rabbit (59, 63), rhesus monkeys (95, 97, 141) and, rarely, in humans (82). The major histological alterations occur in the ileum and colon. The villi appear edematous, swollen, and shortened. Superficial ulceration of the surface epithelium occurs and epithelial cells are disordered and flattened. Crypts are elongated and crypt glands are disrupted with a loss of crypt mucus. Villus goblet cells are devoid of mucus. There is a dense cell infiltration, primarily PMNs, in the villi and lamina propria. Using fluorescent microscopy, Kent et al. demonstrated the presence of Salmonella in the intestinal lumen, scattered in the surface epithelial cells, in the lamina propria and also the submucosa of infected monkeys (95). Takeuchi looked at the penetration of the intestinal mucosa of guinea pigs, preconditioned by starvation and opium treatment (161). He found that when the salmonellae came within close proximity of the intestinal mucosa there was sudden local degeneration of the brush border after which the bacteria gained entry into the epithelium. These observations of invasion of the intestinal mucosa with involvement of both the ileum and the colon are in contrast to what is seen with the enterotoxin diarrheas of V. cholera and E. coli.

The mechanism by which Salmonella attach to the intestinal mucosa has been examined. Duguid et al. studied pili and adhesive properties of Salmonella. He found that piliated strains of Salmonella adhered to epithelial cells while non-piliated strains did not, the only exceptions were cultures of S. sendai which lacked pili but appeared to possess another mechanism for adhering (34). Piliated S. typhimurium had a 25% increase in infectivity when compared to non-pilated cultures (35). Tannock et al. (163) were unable to associate pili, O antigens or flagella with adherence in the mouse ileum. Anti-O serum of S. typhimurium did not reduce in vitro adhesiveness but did prevent colonization of Peyer's patches of the ileum in vivo (84). Further, anti-H serum prevented in vitro adherence, but had no effect in vivo. No definite conclusions can be drawn at this time as to the role of the bacterial cell surface components in colonization and virulence.

Studies on the water and electrolyte transport in the intestines of salmonella infected animals have revealed net ileal secretion of H_2O, K and Cl with decreased absorption of Na in rats (135), monkeys (140), and rabbits (55). Giannella et al. (61) reported high fluid and electrolyte output in humans with salmonellosis. There appears to be at least two possible mechanisms to account for this net

exsorption, namely, (1) altered permeability of the intestinal mucosa damaged by the invasion and inflammation results in a passive loss of fluid and electrolytes, and (2) active secretion occurs in the mucosa, possibly mediated by a toxin. The former mechanism is inconsistent with the fact that certain strains of Salmonella (i.e., SL1027) invade and damage the intestinal mucosa without affecting absorption of Na and Cl (55). Fromm et al. (55) demonstrated absorption of glucose to be normal in rabbits with salmonella diarrhea and that glucose stimulation of Na absorption did occur. No increased permeability to radioactively labeled erythritol, mannitol or albumin was observed when clearance rates in control monkeys were compared to salmonella infected monkeys (66, 97). This is evidence that passive fluid loss is not responsible for salmonella diarrhea. The inflammatory reaction appears to be of some importance in intestinal secretion because it was found that rabbits depleted of neutrophils with nitrogen mustard no longer exhibited salmonella-induced secretion (59). Nitrogen mustard did not alter the response to cholera toxin.

To evaluate the possibility of an enterotoxin mediated secretory process, concentrations of adenylate and cAMP were measured in salmonella infected ileal loops along with cholera toxin stimulated loops (64). An

invasive, fluid evoking strain elicited an increase in adenylate cyclase and cAMP concentration, while strain SL1027 (invasive, non-fluid evoking strain) showed no change. Activation of cAMP appears to be less than maximal since theophylline (which causes an increase in cAMP) enhances the secretory response when administered to salmonella invaded ileum (55). Negligible responses to theophylline were observed in cholera toxin treated ileum (52). Pretreatment of the animals with indomethacin, an anti-inflammatory agent, inhibited fluid secretion and adenylate cyclase activation in salmonella infected animals while cholera toxin exposed animals showed partial inhibition of fluid secretion with no change in adenylate cyclase stimulation (64, 65). Apparently salmonella activation of adenylate cyclase differs from cholera toxin activation of the enzyme.

Salmonella may be stimulating adenylate cyclase activity by some property other than production of an enterotoxin. One proposed mechanism is that the acute inflammatory process attendant to salmonella infection stimulates production of prostaglandins in the gut which activate adenylate cyclase. Observations consistent with this idea include (1) inhibition of the inflammatory response with indomethacin or nitrogen mustard, (2) acute inflammation stimulates synthesis and release of

prostaglandins (83, 169), (3) prostaglandins do cause intestinal secretion (93, 111, 134), and (4) prostaglandins can stimulate adenylate cyclase activity (96). This hypothesis does not explain why invasive S. typhimurium SL1027 fails to evoke a fluid response yet enhances the fluid response to cell-free filtrates of other salmonellae.

While enterotoxin assay systems are being utilized to study Salmonella, the presence of an enterotoxin has not been clearly established. It is not at all certain whether the activities being assayed for represent a true toxin or other virulence factors.

MATERIALS AND METHODS

Bacterial Strains

Salmonella typhimurium strains LGH-3, EWSH-31, BMMC-32, BMMC-59, and *Salmonella oranienburg* BMMC-60 were of human origin and were associated with diarrheal disease. *Salmonella agona* strains E705-78, E709-78, E711-78, E636-78, E695-78, E708-78, E657-78, E649-78, E716-78, E710-78, and E675-78 were isolated from horses and were associated with an outbreak of gastroenteritis in the Michigan State University Veterinary Clinic. *Salmonella infantis* E601-78, and *Salmonella typhimurium* E113-78, E536-78, also obtained from the Michigan State University Veterinary Clinic, were of equine origin and associated with disease in these horses. Equine strains *Salmonella typhimurium* E9288 and *Salmonella anatum* 2CH were obtained from E. V. Morse, School of Veterinary Medicine, Purdue University, Lafayette, Indiana. *S. typhimurium* E9288 was isolated from an acute fatal equine case and *S. anatum* 2CH was isolated from an asymptomatic equine carrier (the clinical history of this patient is published in reference 119). Isolates of *Salmonella typhimurium* 986, 2000, and *Salmonella newport* 1179 and enterotoxigenic *Escherichia coli* H10407 were obtained from J. W. Peterson, University of Texas, Galveston.

Media and Maintenance of Cultures

All cultures used for the study were maintained on agar slants composed of 2.0% peptone (Difco Laboratories, Detroit, Michigan), 0.5% NaCl, and 2.0% agar (47) at 4°C and transferred every 6-10 weeks.

For permanent storage all strains were cultured overnight in 10 ml of Brain Heart Infusion (BHI) broth (Difco Laboratories). BHI cultures were then centrifuged at 12,100 x g for 20 minutes on a Sorvall RC-5 Superspeed Refrigerated Centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). The bacterial cell pellets were washed two times in 0.85% NaCl, added to 5 ml glycerol (Mallinckrodt, St. Louis, Missouri) and frozen at -20°C.

BHI broth was the growth medium used in the enterotoxin assay. Tryptose agar pour plates of appropriately diluted samples were used for viability counts. For experiments on multiplication of Salmonella organisms within the suckling mouse the stomach and entire intestines were removed from each mouse, homogenized in 100 ml sterile distilled water in a Waring blender for two minutes, diluted appropriately, and then plated on MacConkey agar. Lactose negative colonies were counted as Salmonella and the percentage increase in the number of Salmonella present immediately after injection to the number recovered after 2½ hours of incubation was calculated.

Preparation of Culture Supernatants

Organisms from agar slants were inoculated into 5 ml of BHI broth in 150 x 16 mm tubes. Broths were incubated at 37°C for 15-16 hours in a Wheaton Roller Culture Apparatus (Wheaton Scientific, Millville, New Jersey), speed setting one-half rev/min. Cultures were centrifuged at 12,100 x g for 20 minutes on a Sorvall RC-5 Superspeed Refrigerated Centrifuge. The broth supernatants were used as the crude preparations for inoculation of suckling mice, except where filtration is specified.

Filtration and Concentration

When indicated culture supernatants were filtered through 0.45 µm Swinnex-13 membrane filters (Millipore Corporation, Bedford, Massachusetts). The filtrates were assayed directly for enterotoxin activity or were concentrated. Forty-fold concentration was accomplished with an Amicon ultrafiltration unit fitted with a PM30 membrane (Amicon Corporation, Lexington, Massachusetts). Alternatively, the 0.45 µm filters were treated with 1 ml of 35% bovine albumin fraction V (Nutritional Biochemicals Corporation, Cleveland, Ohio) prior to filtration of the culture supernatants to block possible binding of toxin to the filters.

Preparation of Whole Organism Suspensions

When Salmonella organisms were tested for enterotoxin activity sedimented cells of 15 hour BHI roller tube cultures were washed 2-3 times in saline. Cells were resuspended as indicated in either saline, BHI, or culture filtrate to approximately 3 x 10^8 organisms/ml by comparison with the Number 1 McFarland Standard. These preparations were assayed for activity in the suckling mice and plate counts were made for actual viability counts.

Sonication

Sonication of Salmonella organisms was carried on a 100 Watt Ultrasonic Disintegrator (Measuring and Scientific Equipment LTD., London, England), amplitude 7 microns.

Mice

Newborn HA/ICR suckling mice (Harlan Industries, Inc., Cumberland, Indiana) 3 to 5 days old were used in the suckling mouse assay. Mice were separated from their mothers immediately before use and randomly divided into groups of three. Adult HA/ICR mice (Harlan Industries, Inc.) 6 to 8 weeks old were used in the LD_{50} determinations.

Suckling Mouse Assay

The suckling mouse assay was performed as described by Dean et al. (26) with the following modifications. Each

of three mice were injected intragastrically (percutaneously with a 30½ gauge needle) with 0.1 ml of culture preparations containing two drops of 2% Evans Blue dye/ml. After 2½ hours of incubation at room temperature the mice were killed by cervical dislocation. The abdomen was opened and the entire intestine (not including the stomach) was removed with forceps and weighed. The ratio of gut weight to remaining carcass weight was calculated and an average value was obtained for each group of three mice. Activity in the assay is expressed as a ratio of intestinal weight to the remaining body weight (IW/BW). In this study, any values greater than or equal to 0.088 are considered positive responses, values between 0.079-0.087 are indeterminate and any values less than or equal to 0.078 are considered negative. Each experiment was performed at least in duplicate using three mice each time.

LD_{50} Determination in Adult Mice

Salmonella were cultured and washed as described in previous section and resuspended in saline to 3×10^8 organisms/ml. Serial 10-fold dilutions of the suspensions were prepared in saline and each mouse was injected intraperitoneally (IP) with 0.5 ml of the appropriate dilution. Each dilution was injected into five mice. Suspensions were plate counted to determine actual number of viable organisms injected. Death of the mice was recorded over

a period of 14 days. LD_{50} values were calculated by the method of Reed and Muench (136).

Slide Hemagglutination Test

Hemagglutination tests were performed as described by Duguid et al. (34) with the following modifications. Citrated red blood cells from guinea pigs (Colorado Serum Company, Laboratories, Denver, Colorado) were washed three times in saline and made up to a 3% suspension in saline. Alternatively, red cells were washed and resuspended in saline containing 0.5% D-mannose to test for susceptibility of hemagglutination to mannose. Sedimented cells of BHI broth roller cultures were resuspended in 0.25 ml saline. A drop of the bacterial suspension was tested with a drop of suspensions of red cells with and without mannose on a slide. When hemagglutination took place in the suspension without mannose but not in the suspension with mannose, the activity of the strain was recorded as mannose sensitive.

Statistics

The mean of IW/BW ratios ± 1 standard error of the mean for each sample tested was calculated. The student's t-test was used to evaluate results when indicated (12).

RESULTS

Enterotoxin-Like Activity in Culture Supernatants

All human isolates of *Salmonella* (four strains of *S. typhimurium* and one of *S. oranienburg*) elicited positive responses (IW/BW ≥ 0.088) in the suckling mouse assay (Table 1). Of the 16 equine strains studied, six reacted positively. Of these, three were *S. typhimurium*, two were *S. agona* and one *S. infantis*. Negative responses were seen in two of the organisms (IW/BW ≤ 0.078), while the remaining eight isolates were indeterminate. Two *S. typhimurium* and one *S. newport* isolates were obtained from Peterson. He had previously reported production of delayed permeability factor by the *S. typhimurium* isolates. One of these two strains has lost this ability subsequent to three years of laboratory passage. The *S. newport* isolate was negative for the permeability factor. Each of the three strains reacted positively in the suckling mouse assay. An enterotoxigenic strain of *E. coli* was included for control purposes.

Table 1. Production of Enterotoxin-Like Activity in Culture Supernatants

Origin	Serotype	Number Tested	IW/BW ≤ .078	IW/BW .079-.087	IW/BW ≥ .888
Human	S. typhimurium	4	--	--	4[a]
	S. oranienburg	1	--	--	1
Equine	S. typhimurium	3	--	--	3
	S. agona	11	1	8	2
	S. anatum	1	1	--	--
	S. infantis	1	--	--	1
JWP[b]	S. typhimurium	2	--	--	2
	S. newport	1	--	--	1
	E. coli	1	--	--	1

[a]Figures represent number of tested samples with results falling within the range indicated.

[b]Isolates obtained from J. W. Peterson, Galveston, Texas.

Effect of Filter Sterilization on Enterotoxin-Like Activity

Previous studies on E. coli have suggested that filtered and unfiltered culture supernatant fluids react identically in suckling mice (58). In order to verify the presence of a cell-free exotoxin in this study, culture supernatant fluids were filter sterilized by passing through a 0.45 micron Millipore membrane. Results in Table 2 show that positive suckling mouse ratios could not be demonstrated on cell-free filtrates of Salmonella. Activity of enterotoxigenic E. coli was not affected by filtration, a result consistent with the report by Giannella (58).

To investigate the possibility that the toxic factor was being non-specifically bound to the membrane, an attempt was made to prevent binding of a toxin molecule to the filter by saturating the filters with albumin prior to filtration of the supernatants. One ml of 35% albumin solution was passed through the membranes. Subsequently the supernatant fluids of S. typhimurium strains E9288, LGH-3, and E536-78 were also filtered through treated filters. The first 0.5 ml of filtrate was discarded since it served as a wash to remove any albumin retained in the filter apparatus. The next ml of filtrate was injected into suckling mice. Filtrates of albumin treated filters showed no activity in the mice (Table 3).

Table 2. Loss of Enterotoxin-Like Activity After Filter Sterilization of Culture Supernatants

	Sample	Number Tested	IW/BW ≤ .078	IW/BW .079-.087	IW/BW ≥ .088
Salmonella	supernatant	14	--	--	14[a]
	filtrate	14	9	5	--
E. coli	supernatant	1	--	--	1
	filtrate	1	--	--	1

[a]Figures represent number of tested samples with results falling within the range indicated.

Table 3. Effect of Albumin Treatment of Filters Prior to Filter Sterilization of Culture Supernatants

Sample	IW/BW[a] Culture Supernatants	IW/BW[a] Filtrate of Albumin Treated Filters
Broth control	.066 ± .001 (5)	--
S. typhimurium E9288	.089 ± .001 (3)	.073 ± .003 (3)
S. typhimurium LGH-3	.101 ± .001 (2)	.066 ± .002 (2)
S. typhimurium E536-78	.113 ± .006 (2)	.075 ± .003 (2)

[a]Figures represent mean ± 1 standard error of the mean. Number in parentheses indicate the number of separate tests performed.

Effect of Concentration by Ultrafiltration on Enterotoxin-Like Activity of Culture Filtrates

The possibility of a cell bound toxin that is released into the culture medium in very small amounts warranted further investigation. Culture filtrates were concentrated forty-fold by ultrafiltration with a PM30 membrane. The retentates were tested for enterotoxin-like activity. Results in Table 4 indicate that forty-fold concentration had no effect on toxin-like activity of culture filtrates.

Comparison of Culture Supernatants Prepared by Various Centrifugation Procedures

It was determined by plate counts that approximately 4×10^6 organisms were present in 0.1 ml of culture supernatant of S. typhimurium E9288 prepared in the standard manner. Injection of mice with whole culture fluid containing approximately 1.8×10^8 organisms/0.1 ml showed no enhancement in the enterotoxin-like response (Table 5). To determine whether removal of organisms was the factor responsible for loss of activity upon filter sterilization of supernatant fluids it was desirable to remove the organisms from the fluid without altering any other component. The number of bacteria were successfully reduced by centrifuging larger volumes of culture fluid and increasing centrifugation rate and time. A corresponding reduction in enterotoxin-like activity was observed (Table 5).

Table 4. Reactivity of Filtrate Concentrated Forty-Fold by Ultrafiltration With a PM30 Membrane Filter

Sample	IW/BW[a]		
	Culture Supernatant	Filtrate Before Concentration	Filtrate 40X Concentrated
Broth control	.072 ± .001 (6)	--	.074 ± .001 (2)
S. typhimurium E9288	.091 ± .002 (4)	.068 ± .000 (3)	.080 ± .002 (3)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

Table 5. Effect of Variation of Centrifugation Procedure on Enterotoxin-Like Activity of Salmonella

Sample	IW/BW[a]	Count[b]
Broth control	.073 ± .002 (3)	--
S. typhimurium E9288		
Whole culture[c]	.099 ± .002 (3)	1.8×10^8
5 ml culture spun at 12,100 x g for 20 min	.099 ± .003 (5)	4.2×10^6
33 ml culture spun at 12,100 x g for 20 min	.087 ± .002 (2)	2.5×10^5
33 ml culture spun at 27,000 x g for 1 hour	.077 ± .004 (2)	6.05×10^3
S. agona E636-78		
Whole culture	.074 ± .004 (2)	1.6×10^8
5 ml culture spun at 12,100 x g for 20 min	.075 ± .002 (5)	7.7×10^6

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate the number of separate tests performed.

[b]Average number of viable Salmonella/0.1 ml culture fluid.

[c]Whole BHI broth culture was injected directly without prior centrifugation.

A strain of S. agona with a negative suckling mice response was also examined for the effect that the number of bacteria present would have on the activity. Supernatants containing 7.7 x 10^6 organisms/0.1 ml exhibited IW/BW ratios similar to the IW/BW ratios of mice injected with whole cultures with 1.6 x 10^8 organisms in 0.1 ml (Table 5).

Determination of the Number of Viable Salmonella Organisms Required for Enterotoxin-Like Activity

BHI cultures of S. typhimurium E9288 were centrifuged and the supernatant fluid was filter sterilized and used as diluent while the sedimented cells were washed 3-4 times in saline and resuspended in the culture filtrate. Serial dilutions in culture filtrate were prepared from this suspension. Each dilution was plate counted and injected into suckling mice. Table 6 shows that approximately 10^6 organisms are required for enterotoxin-like activity. Preparations containing less than 10^6 organisms show considerably reduced activity.

Enterotoxin-Like Activity Following Heating and Sonication

Culture supernatants of S. typhimurium E9288 and E. coli H10407 were heated in a 100°C or a 60°C waterbath for 30 min, cooled to room temperature and injected into suckling mice. Table 7 shows a loss of activity in both

Table 6. Suckling Mice Activity of Various Doses of S. typhimurium E9288 Organisms

Dose[b]	IW/BW[a]
Filtrate-diluent[c]	.075 ± .002 (4)
1.0×10^7 - 3.0×10^7	.101 ± .003 (2)
5.0×10^6 - 7.0×10^6	.096 ± .006 (3)
2.0×10^6 - 4.0×10^6	.092 ± .005 (4)
1.0×10^6 - 1.5×10^6	.093 ± .003 (2)
5.0×10^5 - 7.0×10^5	.086 ± .002 (3)
1.7×10^5 - 4.0×10^5	.077 ± .003 (3)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate the number of separate tests performed.

[b]Average number of viable Salmonella/ 0.1 ml.

[c]Culture filtrate of S. typhimurium E9288 was used to dilute washed organisms to the indicated concentrations.

Table 7. Effect of Heat on Enterotoxin-Like Activity of S. typhimurium E9288 and Enterotoxigenic E. coli H10407

Sample	IW/BW[a]		
	Culture Supernatant	Culture Supernatant Heated 60°C for 30 min	Culture Supernatant Heated 100°C for 30 min
Broth control	.069 ± .002 (4)	--	--
Salmonella	.094 ± .002 (6)[b]	.074 ± .004 (2)	.072 ± .003 (4)
E. coli	.130 ± .015 (7)	.125 ± .015 (3)	.076 ± .005 (4)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate the number of separate tests performed.

[b]Average number of viable Salmonella in 0.1 ml of these culture supernatant fluids was 9.2×10^6 before heating, after heating at 60°C and 100°C the organisms in the culture fluids were no longer visible.

E. coli and Salmonella at 100°C. E. coli toxin remained active after heating at 60°C while Salmonella supernatant lost enterotoxin-like activity at this temperature. Salmonella supernatants contained 9.2 x 10^6 organisms/0.1 ml before heating and were sterile after 60°C for 30 min.

Whole bacterial cell lysates prepared by sonic disruption failed to induce positive fluid accumulation in the suckling mice (Table 8).

Reactivity of Washed Cells and Reconstituted Filtrates in Suckling Mice

To investigate the possibility that some factor present in culture supernatant fluids was necessary for suckling mice activity, washed cells injected alone were examined. Washed cells of positive strains S. typhimurium E9288 and S. typhimurium LGH-3 as well as cells of two negative strains, S. anatum 2CH and S. agona E636-78 were resuspended to concentration of approximately 3 x 10^7 cells/0.1 ml. Reactivity of the suspensions in suckling mice is shown in Table 9. Washed cells alone of S. typhimurium strains E9288 and LGH-3 were able to elicit a fluid response. S. anatum 2CH and S. agona E636-78 cells were unable to evoke a fluid response.

It was then determined whether the activity of sterile culture filtrates could be restored by adding back organisms. Reconstitution of filtrates with washed cells

Table 8. Effect of Sonication on the Enterotoxin-Like Activity of S. typhimurium E9288

Sample	IW/BW[a]	Count[b]
Broth control	.073 ± .002 (3)	--
Whole culture before sonication	.099 ± .002 (3)	1.8×10^8
Sonicated organisms[c]	.075 ± .002 (3)	1.0×10^5

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate the number of separate tests performed.

[b]Average number of viable organisms/0.1 ml of sample.

[c]BHI broth culture was centrifuged and pelleted bacteria were resuspended in small amount of saline, sonicated and diluted back to original volume in BHI broth, i.e., equivalent to original 1.8×10^8 organisms in 0.1 ml.

Table 9. Restoring Enterotoxin-Like Activity to Filtrates of Salmonella

Sample	IW/BW[a]			
	S. typhimurium E9288	S. typhimurium LGH-3	S. anatum 2CH	S. agona E636-78
Filtrate alone	.081 ± .001 (5)	.072 ± .004 (2)	.070 ± .001 (2)	.073 ± .003 (2)
Washed cells	.089 ± .004 (5)	.098 ± .004 (2)	.072 ± .002 (3)	.071 ± .001 (2)
Filtrate plus E9288 washed cells	.094 ± .004 (2)	.095 ± .005 (3)	.101 ± .001 (2)	.096 ± .001 (2)
Filtrate plus LGH-3 washed cells	.114 ± .004 (2)	.102 ± .001 (2)	.098 ± .009 (3)	.105 ± .006 (2)
Filtrate plus 2CH washed cells	.076 ± .002 (3)	.080 ± .001 (2)	--	.080 ± .001 (4)
Filtrate plus E636-78 washed cells	.074 ± .002 (3)	.077 ± .002 (3)	.081 ± .002 (3)	--

[a]Filtrates represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

to a final concentration of 3×10^7 organisms/0.1 ml did restore the activity. A positive organism could restore the activity to its own filtrate as well as filtrates of another strain. The possibility of an inhibitor in the filtrates of negative strains S. anatum 2CH and S. agona E636-78 was investigated by testing washed cells of positive strains of S. typhimurium E9288 and LGH-3 with filtrates of the negative strains. No inhibition of activity was observed. Washed cells of isolates S. anatum 2CH and S. agona E636-78 had no effect on the activity of filtrates of positive strains.

LD_{50} Determinations of Salmonella in Adult Mice

Evaluation of the suckling mouse model as a measure of pathogenicity of Salmonella isolates was carried out by comparing lethality of positive versus negative suckling mice strains for adult mice. Positive isolates of S. typhimurium E9288 and LGH-3 were found to be at least 500 times more lethal for adult mice than were suckling mice negative isolates S. agona E709-78, S. agona E636-78, and S. anatum 2CH. S. typhimurium E9288 and LGH-3 were at least 40 times more lethal for adult mice than was suckling mice positive S. agona E711-78.

Table 10. LD_{50} Determinations of Salmonella in Adult Mice

Sample	IW/BW[a]	LD_{50}
S. typhimurium E9288	.100 ± .003 (5)	6.4×10^3
S. typhimurium LGH-3	.101 ± .003 (7)	6.9×10^3
S. agona E711-78	.094 ± .005 (7)	$>2.6 \times 10^5$
S. agona E709-78	.079 ± .003 (5)	1.4×10^6
S. agona E636-78	.076 ± .002 (8)	$>3.8 \times 10^6$
S. anatum 2CH	.074 ± .002 (5)	$>3.5 \times 10^6$

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

Mannose Sensitive Hemagglutinating Activity of Salmonella Organisms

Duguid et al. (34) have described mannose sensitive hemagglutination of guinea pig red blood cells as a method for detection of piliated (fimbriated) strains of Salmonella. Piliated strains demonstrated mannose sensitive hemagglutinating activity while non-piliated strains did not. This method was used in the present study in an attempt to correlate suckling mice activity with the presence or absence of pili (Table 11). Of the seven suckling mice positive strains of S. typhimurium examined, six possessed mannose sensitive hemagglutinating activity. None of the isolates of S. agona tested demonstrated hemagglutinating activity,

Table 11. Mannose Sensitive Hemagglutinating Activity of *Salmonella* Organisms

Sample	IW/BW[a]	Mannose Sensitive Hemagglutination[b]
S. typhimurium LGH-3	.101 ± .003 (7)	-
S. typhimurium EWSH-31	.097 ± .008 (2)	+
S. typhimurium BMMC-32	.097 ± .004 (2)	+
S. typhimurium E536-78	.100 ± .001 (8)	+
S. typhimurium E9288	.100 ± .003 (5)	+
S. typhimurium 986	.097 ± .004 (6)	+
S. typhimurium 2000	.092 ± .004 (6)	+
S. agona E705-78	.084 ± .003 (7)	-
S. agona E709-78	.079 ± .003 (6)	-
S. agona E711-78	.094 ± .005 (7)	-
S. agona E636-78	.076 ± .002 (8)	-
S. agona E695-78	.087 ± .003 (10)	-
S. agona E708-78	.085 ± .002 (9)	-
S. anatum 2CH	.074 ± .002 (5)	-
S. newport 1179	.095 ± .005 (5)	+
E. coli H10407	.119 ± .005 (7)	+

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

[b]Hemagglutination of guinea pig red blood cells that took place in a suspension of red cells without mannose but not in that with mannose was recorded as positive mannose sensitive hemagglutination.

regardless of suckling mice reactivity. Suckling mice negative isolate of S. anatum was hemagglutinating negative while suckling mice positive S. newport and enterotoxigenic E. coli exhibited mannose sensitive hemagglutinating activity.

Multiplication of Salmonella Within Suckling Mice

To examine the possibility that the enterotoxin-like activity of Salmonella in suckling mice was related to the ability of the organism to multiply within the suckling mouse gut, the multiplication of a suckling mice positive isolate within the gut was compared to that of a negative strain. In each test the number of viable Salmonella present immediately after injection was determined by plate counts of the gut (intestines and stomach) of each of three mice. Plate counts were made on MacConkey's agar, lactose negative colonies were counted as Salmonella and results of the three mice were averaged. Similar plate counts were made of the gut (intestines and stomach) of three mice after 2½ hr of incubation. The percentage increase in the number of Salmonella during the incubation period was calculated. No statistically significant difference between the suckling mice negative strain and the positive strain was observed (Table 12).

Table 12. Multiplication of *Salmonella* Within Suckling Mice

Sample	IW/BW[a]	Percentage Increase in Number of *Salmonella*[b]
S. typhimurium E9288	.100 ± .003 (5)	113% ± 47% (5)[c]
S. agona E636-78	.076 ± .002 (8)	157% ± 39% (4)[c]

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

[b]Figures represent mean percentage increase in number of *Salmonella* within the mouse gut (including stomach) during 2½ hours of incubation ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

[c]No statistically significant difference.

DISCUSSION

Several studies have shown enterotoxin-like activity for Salmonella in rabbit ileal loops (63, 151), rabbit skin permeability tests (104, 146), CHO cells (147) as well as suckling mice (103). The results of these studies indicate that most clinical isolates of Salmonella react positively in these models although the degree of response varies considerably. In the present study 15 of 25 isolates showed a positive response, 8 were indeterminate while only 2 were clearly negative. S. typhimurium had a higher incidence of reactivity than other serotypes; 100% of human and equine isolates were positive. This is consistent with the findings of Kuhn et al. (104). Some authors report a correlation between degree of host adaptation and enterotoxin-like activity (104, 151). Strains isolated from clinically ill patients evidenced more consistently positive results while food and healthy carrier isolates were more often negative. No attempt was made in this study to correlate suckling mouse reactivity to clinical condition of the patients (see Appendix for brief histories).

Enterotoxins of E. coli and V. cholerae are exotoxins released into broth culture medium during

growth of the organisms. Cell-free preparations of these toxin positive cultures are used to evoke response in the enterotoxin models described. Giannella described positive rabbit ileal loop response with Salmonella only when whole organisms were present; cell-free filtrates and whole cell lysates were not able to evoke fluid accumulation (63). A subsequent report by Sedlock and Deibel demonstrated great enhancement of rabbit loop response to sterile filtrates of broth cultures by pretreatment of intestinal loops with a mucolytic wash. Perhaps the mucus serves as a barrier preventing access of the toxin to the epithelial cells where it presumably would initiate secretion. It has been suggested that normally the toxin can reach its target site only in the presence of an invasive organism which eliminates the barrier (151). Peterson, using skin permeability and CHO cell assays, reports toxin-like activity in cell-free preparations of Salmonella. His studies required the chromatography of culture filtrates on Sephadex G-100 before toxin activity could be demonstrated. It is speculated that this process is eliminating an inhibitor from the filtrates (146, 147). Other investigators were able to assay for the skin permeability factor directly using cell-free filtrates. No inhibitor-like substance was observed (104). Since different growth media were used by the investigators, direct comparison of the data is difficult. Koupal and

Deibel report suckling mouse activity with Salmonella using culture supernatants, no data is shown for the cell-free filtrates of these supernatant fluids (103). When culture supernatants were passed through a filter for sterilization in the present study all of the filtrates of the 14 positive strains that were tested were devoid of activity. Enterotoxigenic E. coli strain used as a control did possess filterable activity in this model. The loss of activity upon filtration of Salmonella was believed to be due to one of two phenomena: (1) the toxin molecule is being non-specifically bound to the membrane during filtration and (2) the organisms present in culture supernatant being removed by filtration are essential to the toxin-like activity in the mice. The former possibility is unlikely in view of the fact that pretreatment of the filters with an albumin solution, which should saturate the binding sites on the filter, had no effect on loss of activity during filtration. Equally ineffective was the attempt to concentrate any small amount of toxin that might be present in the filtrates. Filtrates that were concentrated forty-fold with PM30 ultrafiltration exhibited no activity in the suckling mice. Other authors have found the toxin-like properties reacting in their systems can be retained by a PM30 membrane (146, 152).

To look at the other possibility it was necessary to remove the organisms from the supernatant in such a way as not to destroy a potential toxin molecule. More vigorous centrifugation was the method chosen and the number of bacteria present in supernatant fluids were successfully lowered with a corresponding reduction in toxin-like activity. This is strong evidence that organisms are required for the enterotoxin-like activity of Salmonella in suckling mice.

Diluting S. typhimurium E9288 in filtrate and testing each dilution for suckling mouse activity it was determined that at least 10^6 organisms are required to evoke a positive response (Table 6). Negative activity observed in some Salmonella strains does not appear to be associated with the number of organisms present (Table 5).

Viability of the organisms was altered to study the role viability plays in enterotoxin-like activity of Salmonella. Heat and sonication were the methods employed to reduce viability. Temperature studies on S. typhimurium E9288 were performed in parallel with enterotoxigenic E. coli. Salmonella viability was lost at temperatures of 60°C and 100°C for 30 min as was enterotoxin-like activity (Table 8). ST of E. coli was sensitive to heating at 100°C for 30 min, but was stable at temperatures of 60°C (Table 8) and 80°C (data not shown). Some authors report only a

slight decrease in E. coli toxin activity after heating at 100°C for 15-30 min (26, 58, 89, 116) while other authors have observed a marked reduction in response with this treatment (77, 88, 120).

Whole bacteria lysates of S. typhimurium E9288, prepared by sonic disruption had negative reactivity in suckling mice. Approximately 10^8 lysed bacteria injected into the mice did not evoke a response. Intact and live bacteria seem to be required for enterotoxin-like activity of Salmonella in suckling mice. A loss of enterotoxin-like activity is observed with destruction of viability as demonstrated by the loss of activity upon heating and sonication.

According to reports by Giannella (63) and Koupal and Deibel (103) washed organisms alone evoked an enterotoxic response. Washed cells of positive cultures reacted positively while cells of negative isolates elicited no response. Sedlock and Deibel reported that the presence of an invasive, non-fluid evoking strain dramatically enhanced rabbit ileal loop activity of sterile filtrates of positive strains in ileal loops not pretreated with the mucolytic agent. It was desired to know if such a phenomenon could be observed in this system since this would indicate that a toxic factor was present in culture filtrates which requires organisms to demonstrate activity.

Table 9 shows results obtained by mixing filtrates and organisms of different strains. No enhancement of response of filtrates could be observed in the presence of negative cells. Likewise, activity of filtrates of negative strains could be converted to positive in the presence of a positive strain ruling out the possibility of an inhibitor present in filtrates of negative strains. This is further evidence that organisms are the active agent in this system.

Since the suckling mouse system does not seem to be assaying for an enterotoxin with Salmonella, at least not a toxin analogous to ST or CT, it was of interest to determine whether this might be used as a measure of microbial virulence. LD_{50} results suggest that it is useful for that purpose in that suckling mice positive strains were significantly more virulent for adult mice than were negative strains, with the exception of the positive strain of S. agona E711-78.

Throughout this study some variability of the response of suckling mice to Salmonella was observed. Positive Salmonella and negative broth controls were performed with each assay and only data collected in assays with valid controls were used in this investigation. Some strains of S. agona that reacted positively initially were no longer positive when examined a year later. Presumably the toxic or virulent principle was lost upon

laboratory passage. Similar observations have been made by Peterson (personal communication) while Sedlock and Deibel have suggested the enterotoxic characteristic to be stable in Salmonella (151). In this study both stable suckling mice positive strains and Salmonella strains not stable for suckling mice activity were observed.

The factor(s) responsible for the suckling mice activity and/or variability as yet remains undefined. Properties of the Salmonella organisms were examined to find a possible correlation of a known virulence factor with suckling mice activity. Factors considered were LPS, Vi antigen, capsules, and pili. Endotoxin does not appear to be the agent responsible for suckling mice activity since sonicates which are rich in LPS are negative in suckling mice. Further, heating would not destroy LPS, but it destroys activity. Also negatively reacting Salmonella organisms contain LPS yet still are negative in the suckling mice.

All positively reacting Salmonella isolates were examined for the presence of Vi antigen. None of the strains agglutinated in Vi antisera.

Capsule stains were performed on several isolates of both suckling mice positive and negative Salmonella. Capsules could not be demonstrated on any of the isolates examined.

Mannose sensitive hemagglutination of guinea pig red blood cells was used to determine piliated strains from non-piliated isolates. There appears to be a correlation between the presence of pili as determined by this method and enterotoxin-like activity, 78% of suckling mice positive strains examined possessed mannose sensitive hemagglutinating activity. None of the suckling mice negative or indeterminate strains were found to be piliated as determined by this method. In an attempt to correlate the presence of pili with microbial virulence for adult mice it was learned that of the two more virulent strains of S. typhimurium (by LD_{50} determinations) only one, S. typhimurium E9288 possessed mannose sensitive hemagglutinating activity. Less virulent strains of S. agona and S. anatum exhibited no hemagglutinating activity. It is difficult to make any correlations between the presence of pili and virulence for adult mice at this time.

O and H antigens of each serotype were examined for a possible correlation of certain antigens wich suckling mice activity since serotype S. typhimurium appears to be more active in suckling and adult mice whereas S. agona and S. anatum appear less active. Antigens are listed in Appendix, Table 16; however, no correlation seems to be immediately evident.

The ability of a given strain to multiply within the gut of the suckling mouse host does not appear to be responsible for the enterotoxin-like activity of Salmonella in suckling mice. The increase in the number of organisms of a suckling mice negative strain of Salmonella within the mouse gut was similar to the increase observed with a positive isolate.

Suckling mice enterotoxin-like activity can be observed with Salmonella. It appears that this activity requires the presence of at least 10^6 viable organisms. A cell-free toxin could not be demonstrated. The suckling mouse assay exhibited a considerable degree of variability with some strains throughout this study. However, a correlation between suckling mice reactivity and virulence for adult mice as well as a correlation between suckling mice response and the presence of pili as determined by the mannose sensitive hemagglutinating activity are suggested.

APPENDIX

Table 13. Organism, Source of Isolation, and Disease Association

Origin	Case Number	Species	Source/Diagnosis
Human	LGH-3	S. typhimurium	Feces/gastroenteritis
	EWSH-31	S. typhimurium	
	BMMC-32	S. typhimurium	Feces/ulcerative colitis
	BMMC-59	S. typhimurium	Blood/enteric fever, diabetes
	BMMC-60	S. oranienburg	Feces/diarrhea 1 month
Equine	E705-78	S. agona[a]	Kidney, liver, lung/ possible congenital immunodeficiency
	E709-78	S. agona	Rectum/post-operative and post-gentamycin profuse watery diarrhea
	E711-78	S. agona	Feces/pneumonia and diarrhea
	E636-78	S. agona	Rectum/possible pneumonia
	E695-78	S. agona	Feces/pneumonia and diarrhea
	E708-78	S. agona	Rectum/post-operative diarrhea
	E657-78, E675-78	S. agona	Feces/chronic diarrhea
	E649-78	S. agona	Feces/splint surgery

[a] S. agona isolates were associated with a diarrheal outbreak at a veterinary clinic.

Table 13--Continued

Origin	Case Number	Species	Source/Diagnosis
Equine	E716-78	S. agona	Colon/3 days post-operative diarrhea
	E710-78	S. agona	Rectum/congenital tendon defect, diarrhea
	E113-78	S. typhimurium	Feces/diarrhea
	E536-78	S. typhimurium	Liver, kidney, intestine/diagnosis undetermined
	E601-78	S. infantis	Intestine/diagnosis undetermined
	2CH	S. anatum	Feces/asymptomatic carrier
	E9288	S. typhimurium	Feces/fatal
JWP[b]	986	S. typhimurium	Positive for rabbit skin permeability factor, lost activity upon subculture
	2000	S. typhimurium	Positive for rabbit skin permeability factor
	1179	S. newport	Negative for rabbit skin permeability factor
	H10407	E. coli	Enterotoxigenic strain

[b]Isolates obtained from J. W. Peterson, Galveston, Texas. Permeability factor activity reported here was performed in his laboratory.

Table 14. Production of Enterotoxin-Like Activity in Culture Supernatants

Origin	Case Number	Species	IW/BW[a]
Human	LGH-3	S. typhimurium	.101 ± .003 (7)
	EWSH-31	S. typhimurium	.097 ± .008 (2)
	BMMC-32	S. typhimurium	.097 ± .004 (2)
	BMMC-59	S. typhimurium	.113 ± .010 (3)
	BMMC-60	S. oranienburg	.108 ± .004 (2)
Equine	E705-78	S. agona	.084 ± .003 (7)
	E709-78	S. agona	.079 ± .003 (6)
	E711-78	S. agona	.094 ± .005 (7)
	E636-78	S. agona	.076 ± .002 (8)
	E695-78	S. agona	.087 ± .003 (10)
	E708-78	S. agona	.085 ± .002 (9)
	E657-78	S. agona	.088 ± .005 (6)
	E649-78	S. agona	.083 ± .003 (7)
	E716-78	S. agona	.083 ± .004 (8)
	E710-78	S. agona	.084 ± .003 (9)
	E675-78	S. agona	.084 ± .004 (7)
	E113-78	S. agona	.098 ± .001 (5)
	E536-78	S. typhimurium	.100 ± .001 (8)
	E601-78	S. infantis	.102 ± .003 (4)
	2CH	S. anatum	.074 ± .002 (5)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

Table 14--Continued

Origin	Case Number	Species	IW/BW[a]
Equine	E9288	S. typhimurium	.100 ± .003 (5)
JWP[b]	986	S. typhimurium	.097 ± .004 (6)
	2000	S. typhimurium	.092 ± .004 (6)
	1179	S. newport	.095 ± .005 (5)
	H10407	E. coli	.119 ± .005 (7)
	Broth control		.073 ± .002 (9)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

[b]Isolates obtained from J. W. Peterson, Galveston, Texas.

Table 15. Loss of Enterotoxin-Like Activity After Filter Sterilization of Culture Supernatants

Sample	IW/BW[a] Supernatant	Sterile Filtrate
S. typhimurium LGH-3	.101 ± .003 (7)	.072 ± .002 (4)
S. typhimurium EWSH-31	.097 ± .008 (2)	.068 ± .004 (2)
S. typhimurium BMMC-32	.097 ± .004 (2)	.071 ± .001 (2)
S. typhimurium BMMC-59	.113 ± .010 (3)	.076 ± .004 (2)
S. oranienburg BMMC-60	.108 ± .004 (2)	.084 ± .004 (2)
S. agona E711-78	.094 ± .005 (7)	.079 ± .001 (2)
S. agona E657-78	.088 ± .005 (6)	.079 ± .000 (2)
S. typhimurium E113-78	.098 ± .001 (5)	.072 ± .008 (2)
S. typhimurium E536-78	.100 ± .001 (8)	.072 ± .002 (3)
S. infantis E601-78	.102 ± .003 (4)	.082 ± .004 (2)
S. typhimurium E9288	.100 ± .003 (5)	.073 ± .002 (4)
S. typhimurium 986	.097 ± .004 (6)	.080 ± .006 (3)
S. typhimurium 2000	.092 ± .004 (6)	.073 ± .006 (2)
S. newport 1179	.095 ± .005 (5)	.075 ± .008 (2)
E. coli H10407	.119 ± .005 (7)	.119 ± .006 (2)

[a]Figures represent mean ± 1 standard error of the mean. Numbers in parentheses indicate number of separate tests performed.

Table 16. Summation of Properties of Various Serotypes of Salmonella

Serotype	IW/BW ≥ .088	Mannose Sensitive Hemagglutination	Antigens		
			O	H	
				Phase 1	Phase 2
S. typhimurium	9/9[a]	6/7[b]	1,4,12(5)	i	1,2
S. agona	2/11	0/6	1,4,12	f,g,s	--
S. anatum	0/1	0/1	3,10	e,h	1,6
S. newport	1/1	1/1	6,8	e,h	1,2

[a]Figures represent number of isolates with IW/BW ≥ .088 over the number tested.

[b]Figures represent the number of isolates with mannose sensitive hemaglutinating activity over the number tested.

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