Pathogenesis of Salmonellosis

STUDIES OF FLUID SECRETION, MUCOSAL INVASION, AND MORPHOLOGIC REACTION IN THE RABBIT ILEUM

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ABSTRACT Strains of *Salmonella typhimurium* were studied in the ligated rabbit ileal loop model to gain insight into the mechanisms whereby bacteria which invade the gastrointestinal mucosa evoke fluid exsorption. The organisms employed differed in various biologic attributes including the ability to invade the ileal epithelium, multiply within the mucosa, elicit an acute inflammatory reaction, and disseminate across the intestinal wall. Some strains provoked small intestinal fluid exsorption although these did not elaborate enterotoxin. Only those strains which invaded the mucosa were accompanied by either mucosal inflammation or fluid exsorption. Noninvasive strains produced neither histologic abnormalities nor fluid secretion. While strains which invaded the mucosa caused an acute inflammatory reaction, not all such strains evoked fluid secretion. Furthermore, there was no correlation in ability of invasive organisms to evoke fluid secretion or in the intensity of mucosal inflammation, number of intramucosal salmonellae, or in ability to disseminate from the rabbit ileum.

These observations suggest that, as is the case in shigellosis, mucosal invasion may be a necessary factor for the intestinal fluid loss in salmonellosis. A bacterial property or factor, in addition to invasion of the gastrointestinal mucosa, seems to be responsible for fluid exsorption. However, it is unlikely that a salmonella enterotoxin comparable to that elaborated by *Vibrio cholerae,* toxigenic *Escherichia coli,* or *Shigella dysenteriae* 1 is related to fluid secretion in salmonellosis.

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INTRODUCTION

The study of acute diarrheal disease, and in particular that caused by bacteria, has intensified because of the current cholera pandemic and the recent Shiga dysentery epidemic in Central America. Two major categories of bacterial diarrhea are presently recognized (1–3). In the first, the so-called toxigenic diarrheas, an enterotoxin is elaborated in the lumen of the small intestine evoking active fluid secretion by the intestinal epithelium (1–3). This type is best exemplified by the disease caused by the noninvading *Vibrio cholerae* and by some strains of *Escherichia coli.* Enterotoxin action on the intestine is believed to be mediated via stimulation of the mucosal adenyl cyclase-cyclic/AMP system (2–6). In the second category of acute bacterial diarrhea, invasive organisms, as exemplified by shigellae, salmonellae, and some *E. coli,* provoke diarrhea but no enterotoxins have yet been described with the exception of *Shigella dysenteriae* 1 (1–3, 7). These organisms invade and multiply within the intestinal mucosa, causing mucosal damage and fluid loss (7–9). While progress in elucidating the mechanism of intestinal fluid loss in the toxigenic diarrheas has been substantial, little is known of the mechanism of fluid entrance into the bowel, i.e., exsorption,[1] evoked by the invasive organisms.

Our approach to defining the mechanism of fluid exsorption caused by invasive bacterial enteropathogens was to utilize the rabbit ileal loop model and various strains of *Salmonella typhimurium* which possessed dif-

[1] For convenience, fluid accumulation in ligated intestinal loops is referred to as exsorption or secretion. No specific mechanism of fluid production is implied by the use of these terms.

TABLE I

Properties of Salmonella typhimurium Strains in Various Animal Systems

Strain	Mouse LD_{50} (log_{10})	Guinea pig LD_{50} (log_{10})	Diarrhea in monkeys (no. positive/no. tested)
W118	1.5	<2.0	13/19
TML	1.0	<2.0	20/25
M206	8.5	>8.0	0/5
SL 1027	5.0	5.0	1/13
LT-7	6.0	ND*	ND
PG-41	7.4	ND	ND
9SR[2]	7.5	ND	ND
THAX-1	>8.0	ND	ND

* ND, not done.

ferent biologic attributes. In the present study, we correlated observations on fluid secretion,[1] mucosal invasion and dissemination of organisms, and the mucosal response as seen microscopically. We also examined the possibility that salmonellae may elaborate an enterotoxin which could promote fluid exsorption, a consideration prompted by our study of three adult cases of salmonellosis with fulminant, cholera-like diarrhea (10, 11). We sought to determine the relationship of mucosal invasion by salmonellae to the mucosal inflammatory response and fluid production by the rabbit ileum.

METHODS

Bacterial strains. Eight strains of *Salmonella typhimurium* were employed in this study. The properties of these strains are summarized in Table I. Four strains (W118, TML, M206, and SL 1027) were used in all of the protocols to be described, with selected studies performed on the additional strains LT-7, PG-41, 9 SR-2, and Thax-1. Strain W118, used in previous experiments (9, 12–17), was originally isolated from a human case of salmonellosis; TML was isolated from a patient with salmonellosis with fulminant, cholera-like diarrhea (10, 11). Two standard laboratory strains, M206, with the property of impaired ability to multiply and survive within macrophages (18, 19) and SL 1027 (20), a genetically marked *Salmonella typhimurium* LT-2 strain, were included for comparative reasons. All strains behaved serologically and biochemically as typical *Salmonella typhimurium*. These strains were maintained in the lyophilized state and fresh ampoules were opened for each series of studies so that multiple studies could be done from the same clone. Strains were grown overnight in brain-heart infusion (BHI) [2] broth, cells sedimented by centrifugation, washed in sterile isotonic saline, and resuspended in fresh BHI broth to the desired concentration. Osmolality of the BHI broth-salmonella inoculum was 337.9±1.5 mosm/kg (mean ±SE, $n = 12$).

Rabbit ligated loop studies. New Zealand strain adult albino rabbits weighing 1.5–2.5 kg were housed individually and food withheld for 48 h prior to study. The tenets of the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council were observed. The technique of preparation of the rabbits, construction of ligated ileal loops, and of inoculation is as described previously (21) but was modified as follows: only one test loop was constructed per animal with an intervening blank loop and broth control loop. All loops were approximately 20 cm long, and 1 ml of the appropriate sample was inoculated into the ligated loop. Preliminary studies revealed that position of the test loop did not influence the results. Groups of animals were sacrificed at 3, 5, 7, 12 and 18 h with intravenous pentathol. Among the 280 rabbits employed, occasional animals demonstrated positive reactions in control loops; these animals were excluded from analysis.

Fluid secretion. At each time interval, the length of loop was measured and its contents emptied by gravity into a graduated cylinder to determine volume. Results are expressed as volume: length ratio to correct for variability in loop length between animals. Data are expressed as mean ±1 SE ml/cm and were analyzed for statistical significance using the Student's t test (22).

Histological and fluorescent antibody studies. At each time interval, portions of both test and control loops were fixed in 10% buffered formalin and processed for light microscopy being stained with hematoxylin-eosin and with Giemsa stains. Slides were coded and read by one of the authors (G. J. D.) without knowledge of experimental details. Contiguous portions of both loops were fast frozen in liquid isopentane and examined by the fluorescent microscopy technique. These tissues were usually examined within 7 days of sacrifice and at least five animals at each time interval were so studied. The details of the preparation of the anti-salmonella antisera, conjugation to fluoroscein, and processing of tissues have been described previously (23).

Bacteriology and dissemination of organisms across the intestine. At time of sacrifice, and prior to handling the intestine, samples of heart blood, and portions of liver and spleen were cultured for salmonellae. In addition, the number of salmonellae in loop fluid was quantitated by serial dilution of an aliquot of the loop fluid and plating, in duplicate, on MacConkey agar. All isolations were confirmed by agglutination with specific antiserum and selected biochemical tests.

Examination of cell-free products. Several preparations from strains W118 and TML were tested. One consisted of sterile supernatant fluid of syncase broth cultures that were vigorously shaken for 16 h at 37°C. The second preparation was sterile supernatant fluid obtained by sonic disruption of a 10% (volume for volume) aqueous suspension of bacteria. In addition, two other preparations of strain TML were also tested. 1 ml of whole culture, containing 10^9 viable cells, was inoculated into a ligated loop of eight animals. Animals were sacrificed at 18 h, loop fluid was collected in iced tubes, and the fluid from all eight animals was pooled, concentrated 50-fold by pressure dialysis, and sterilized by filtration. A 1 ml portion of the resultant sterile fluid was inoculated into ligated ileal loop of two animals and loop reaction read at 18 h. The last preparation of TML was a Van Heynigen preparation (24) of organisms grown in syncase broth for 72 h. Tissue samples of all loops were taken for histological examination.

LD_{50} determinations in mice. Balb-c strain mice weighing 16–18 grams were housed five per cage. Bacterial strains were grown in broth for 18 h, and serial 10-fold dilutions made in isotonic saline. 0.5 ml of each dilution (10^9–10 organisms) were injected intraperitoneally in groups of 10

[2] *Abbreviations used in this paper:* BHI, brain-heart infusion; PMN, polymorphonuclear leukocyte.

mice each. Deaths were recorded over a period of 21 days. LD_{50} values were calculated by the method of Reed and Muench (25).

Oral infection of guinea pigs. Walter Reed or Hartley strain guinea pigs weighing 250-350 g were fasted for 4 days but allowed water. The challenge dose of bacteria suspended in 10 ml of BHI broth was administered by stomach tube and 1 ml of tincture of opium was injected intraperitoneally immediately after challenge. Deaths were recorded over a period of 21 days after challenge. The details of this model have been published previously (12). LD_{50} values were calculated by the method of Reed and Muench (25).

Oral infection of Rhesus monkeys. Rhesus monkeys (*Macaca mulatta*) weighing 2–3 kg were fasted overnight but allowed water. Without anesthesia, they were fed 5×10^{10} agar-grown organisms suspended in 20 ml of broth by stomach tube. Monkeys were examined twice daily for 14 days for evidence of diarrhea and stools were cultured daily. Diarrhea, defined as more than one liquid stool for at least 2 consecutive days, usually was evident in 24–72 h after challenge.

RESULTS

Mucosal invasion by *Salmonella typhimurium* and morphologic consequences

All eight strains were studied in the rabbit ileal loop model. Strains 9SR-2 and Thax-1 did not invade the mucosa, no organisms being seen in the mucosa with either fluorescent antibody microscopy or in Giemsa-stained sections and neither produced any detectable morphologic abnormalities. The six remaining strains (Table I) invaded the intestinal mucosa but three distinct morphologic patterns were observed. Strains W118 and TML behaved identically and will be described first.

W118 and TML. At 3 h, salmonellae were seen in the lumen, adherent to the brush border, and occasionally within villus epithelial cells. Villus goblet cells were devoid of mucus. At 7 h, larger numbers of organisms were seen on and in epithelial cells. Invasion was neither accompanied by epithelial cell destruction nor by an inflammatory reaction in the vicinity of invasion. However, although invasion usually occurred in the upper third of the villus, a focal circumscribed acute inflammatory lesion—resembling a microabscess—was seen in the basilar portions of approximately 5% of villi. This lesion was comprised of large numbers of polymorphonuclear leukocytes (PMN's) and was associated with a marked distortion of the overlying epithelium (Figure 1*A*). Clumps of salmonellae were seen attached to the brush border and many appeared to be entering the villus tip in the extrusion zone and propagating there (Figure 2*A*). Clumps of bacilli were frequently seen in the lamina propria (Figure 2*B*). A striking feature was the relatively greater involvement of the epithelium overlying lymphoid aggregates associated with an acute inflammatory infiltrate of the lymphoid tissue itself (Figure 3*A*). Moderate numbers of bacilli appeared to be multiplying both within cells and in the interstitial tissue and this was accompanied by lymphocyte blast transformation (Figure 3*B*). At 12 and 18 h, salmonellae were widely scattered throughout the epithelium, lamina propria (Figure 4*B*), and even occasionally being seen within lacteals which were occasionally dilated. PMN's, containing organisms, were seen in the epithelium and in the lumen. By 18 h, most villi were blunted, swollen, the lamina propria edematous and massively infiltrated with PMN's. The villus epithelium was disordered, cuboidal to low columnar, and hypercellular. Salmonellae were diffusely scattered throughout the mucosa. The crypts were hyperplastic, increased in depth and with numerous mitoses but well preserved with retention of mucus and without an increase in crypt lumen diameter (Figure 5*A*). Mucosal blood vessels appeared normal.

M206. The pattern of mucosal invasion of strain M206 and the pathologic lesion differed from that described above (contrast Figures 1*A* and 1*B*, 4*A* and 4*B*, and 5*A* and 5*B*). At 3 h, sheets of organisms were present in the lumen, but very few were attached to the brush border. Unlike the above described strains, the number of organisms within the mucosa was few and with increasing time after infection, the number of salmonellae attached to the epithelium or within the mucosa did not increase. At 18 h only occasional single bacilli were seen within the mucosa. No morphologic abnormalities were apparent until 7 h when a few PMN's were seen in crypt areas and in the base of the villus core (Figure 1*B*). At 12 h, little progression had occurred with only approximately 5% of the villi being abnormal. Organisms were seen within the PMN's in the lamina propria and in lymphoid aggregates as well as free within the lamina propria but far fewer in number than observed with strains W118 and TML (Figure 4*A* and 4*B*). At 18 h, the mucosa was intact without the focal necrotizing lesions, disordered villus epithelium, hemorrhage or dense infiltration of PMN's or crypt hyperplasia that characterized the lesions produced by strains W118 and TML (Figure 5*A* and 5*B*). Although our methods do not allow precise quantitation of the number of organisms either attached to or invading the mucosa, it appeared that strain M206 both attached and invaded in far fewer numbers and did not multiply and survive within the mucosa as did strains W118 and TML.

Other strains. Strains SL 1027, LT-7, and PG-41 were only studied at 18 h and were unlike the three above described strains. The morphologic abnormalities appeared intermediate between those produced by strains W118 or M206. An acute inflammatory reaction was evident, more intense in the mucosa overlying lymphoid aggregates, but diffusely involving 30–50% of villi. There was no goblet cell mucus discharge, crypt hyperplasia, or marked abnormality of the villus epithelium.

Dissemination of organisms from ligated loops (Table II)

No organisms were recovered from heart, liver, or spleen at 18 h with strains 9SR-2 and Thax-1. On the other hand, strains W118 and TML penetrated the intestinal wall and could be cultured from the liver and spleen in some animals as early as 3 h. By 18 h, salmonellae were cultured from liver and spleen in most ani-

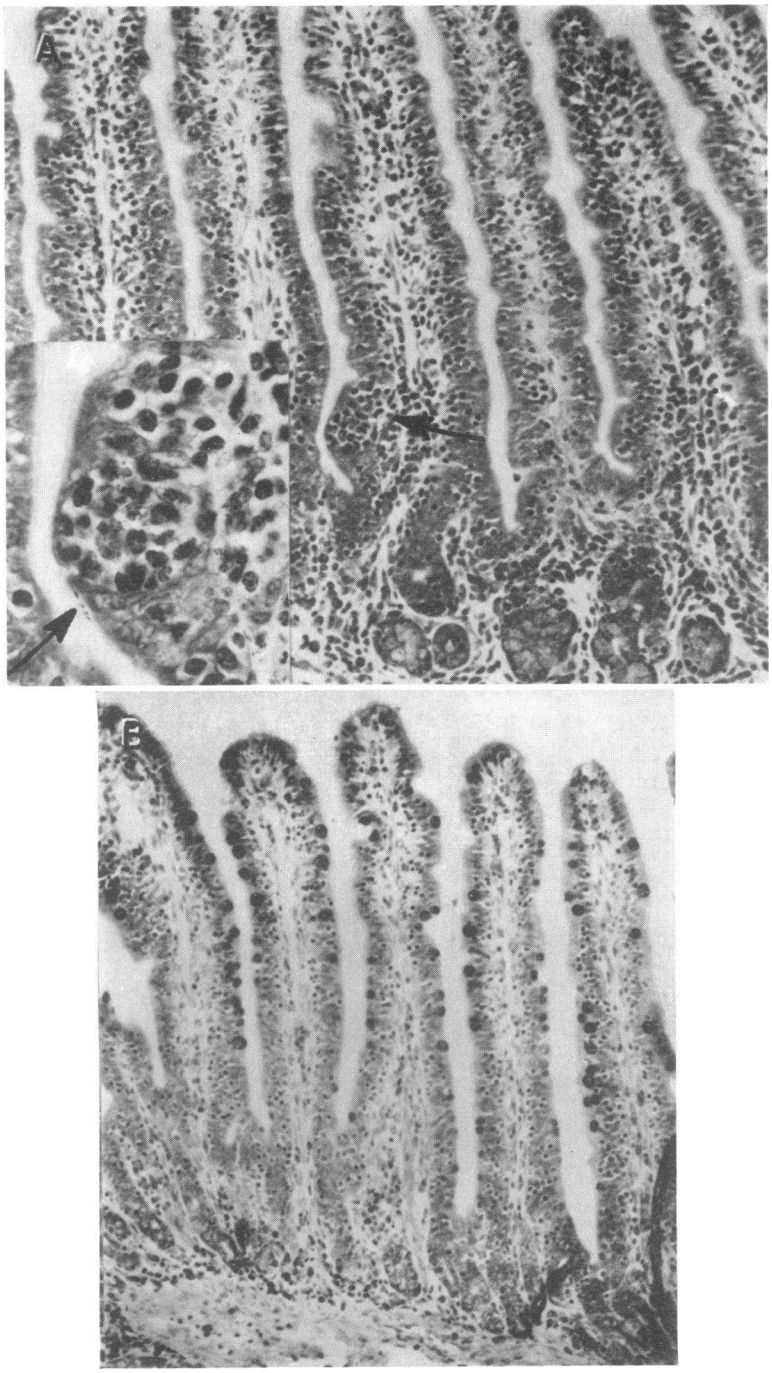

FIGURE 1 Ileal mucosa 7 h after inoculation. (*A*) Strain TML. Normal villus epithelium and architecture except for absence of goblet cell mucus and focal inflammatory lesion (arrow) at base of central villus (Giemsa × 130). *Inset:* High power view of focal inflammatory lesion containing PMN's and demonstrating bacilli (arrows) attached to disordered and attenuated surface epithelium (Giemsa × 1,000). (*B*) Strain M206. Normal mucosa except for a few PMN's at base of villi. Note abundant goblet cell mucus (Giemsa × 130).

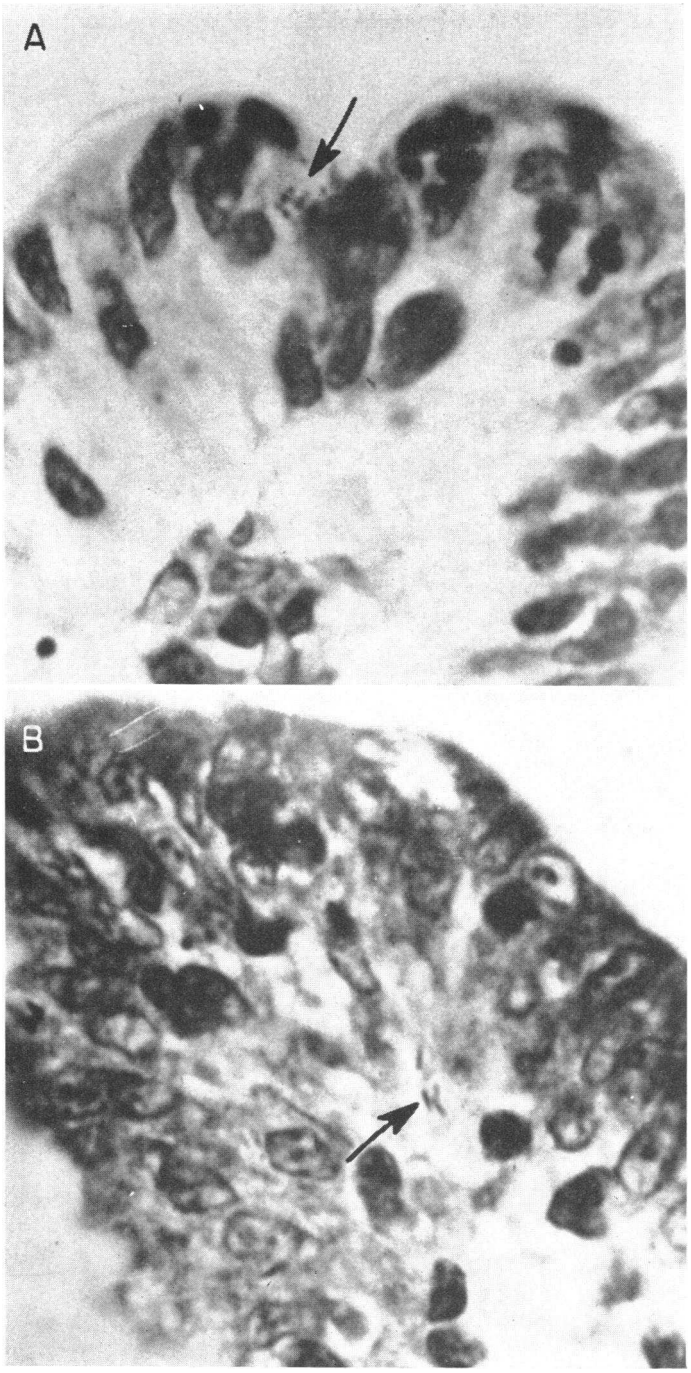

FIGURE 2 High power view of villi with strain TML 7 h after inoculation. (*A*) Salmonellae (arrow) within epithelial cells in extrusion zone (Giemsa × 1,000). (*B*) Salmonellae within lamina propria (Giemsa × 1,000). Note lack of cellular damage or inflammation.

mals and 25% of the animals demonstrated positive heart blood cultures. In contrast, strain M206 demonstrated a distinctly different pattern. Although salmonellae could be cultured from most livers and two of six spleens as early as 3 h, only a single spleen was positive at 12 hours and 83 other cultures, at 7, 12, and 18 h were negative. M206 was never cultured from heart blood at any time interval examined. Strains SL 1027, LT-7, and PG-41 disseminated across the intestinal wall and at 18 h were recovered from liver or spleen, i.e., SL 1027 from 78%, LT-7 from 20%, and PG-41 from 50% of liver and spleen cultures, respectively.

Fluid secretion

The noninvasive strains 9SR-2 and Thax-1 did not elicit fluid secretion nor did invasive strains SL 1027, LT-7, or PG-41. The three remaining invasive strains, W118, TML, and M206, caused a positive loop reaction with secretion of fluid (Table III). Measurable fluid secretion was detected at 3 h, the earliest time interval examined (fluid secretion significantly greater than control, $P < 0.01$, for each organism). There were no differences in fluid secretion at 3, 5, or 7 h. Fluid secretion

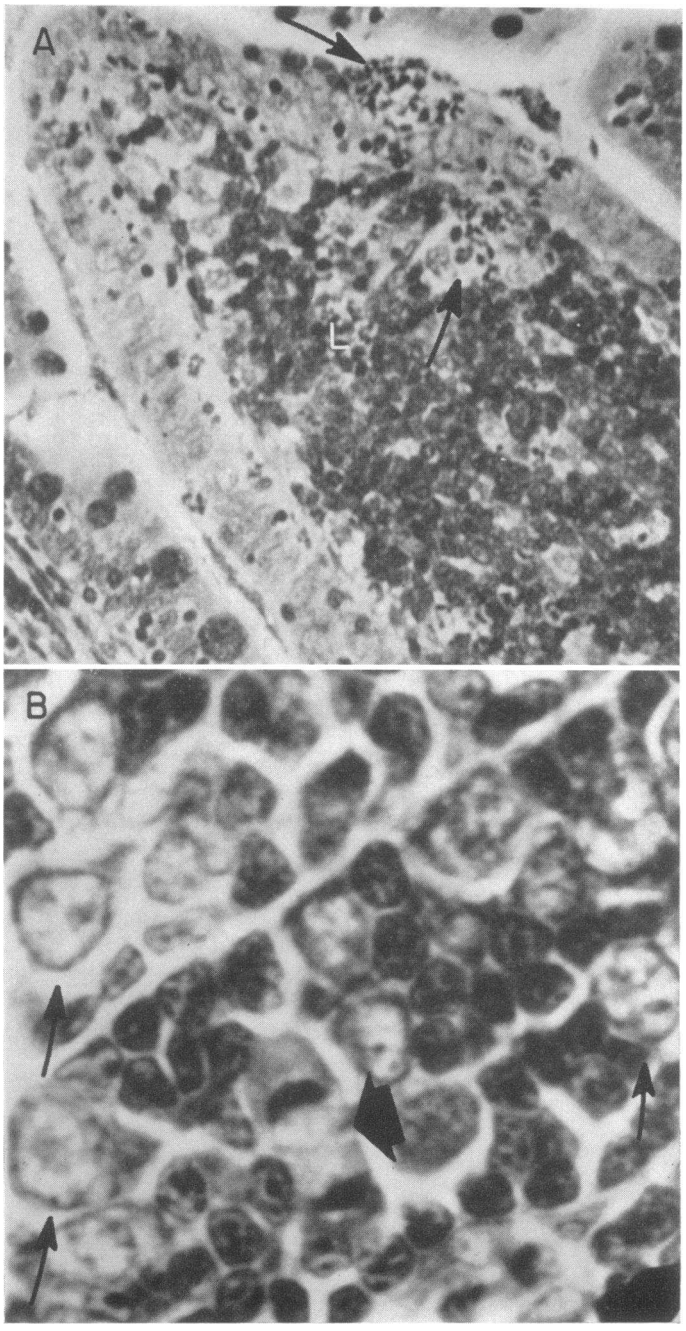

FIGURE 3 Ileal mucosa 7 h after inoculation with strain TML. (*A*) Epithelium overlying lymphoid tissue (L). Note acute inflammatory reaction involving surface epithelium (arrow) and lymphoid (arrow) tissue (Giemsa × 450). (*B*) High power view of lymphoid aggregate. Note immature lymphoblasts (small arrows) and mitotic figure (large arrow) in center (Giemsa × 1000).

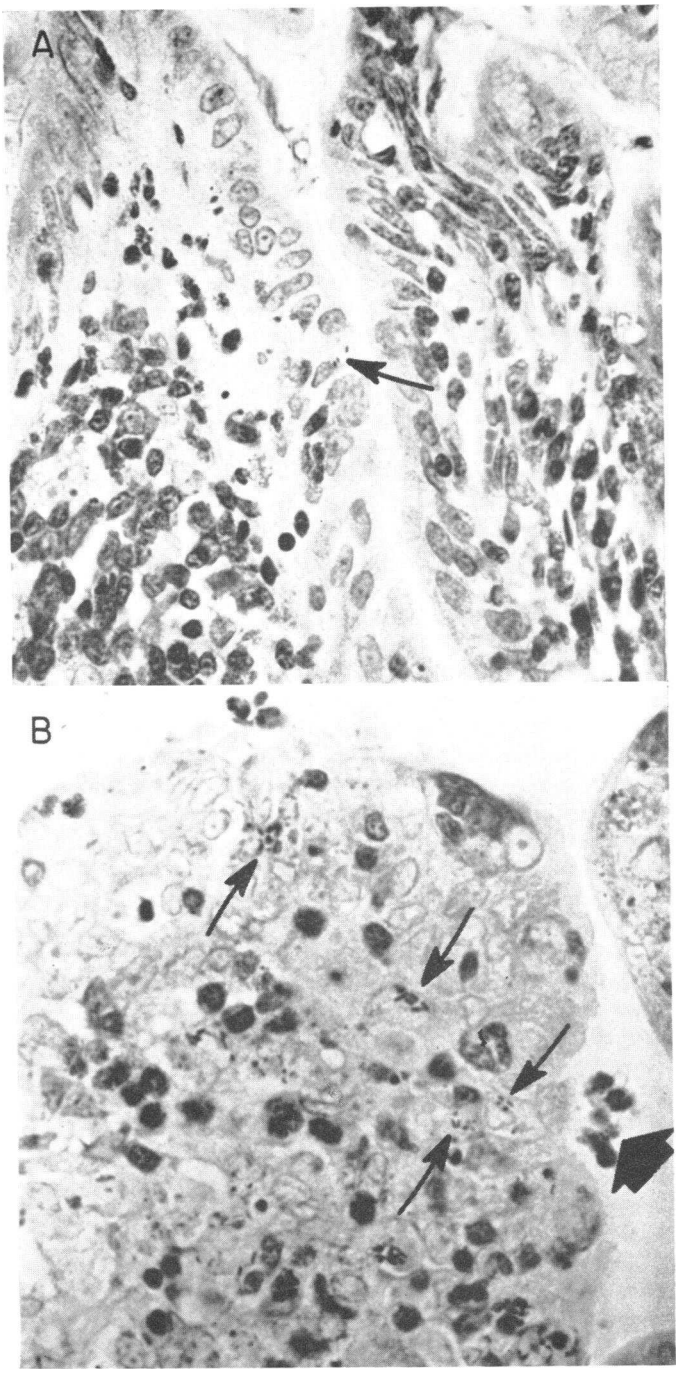

FIGURE 4 Ileal mucosa 12 h after inoculation. (*A*) Strain M206. Note three intraepithelial bacilli (arrow) and PMN's in lamina propria (Giemsa × 1,000). (*B*) Strain TML. Large clumps of bacilli (small arrows) are scattered throughout villus. PMN's also abundant throughout villus and extruding (large arrow) into lumen (Giemsa × 1,000).

increased at 12 h, with the exception of strain M206, and further increased at 18 h such that the volume at 18 h was significantly greater, $P < 0.01$, than that at either 3, 5, or 7 h for each of the three organisms.

While a strict dose-response curve could not be constructed, as few as 100 inoculated organisms elicited fluid secretion ($P < 0.01$ compared with control loops) which tended to increase in response to increasing in-

oculum size (Table IV). Although there were no differences in fluid secretion between 10^2, 10^4, or 10^5 organisms, the volume of fluid secreted in response to 10^9 organisms was significantly greater than that with 10^2 organisms (W118 $P < 0.05$, TML and M206 $P < 0.01$).

A summary of the invasive and fluid provoking properties of each of the eight strains is shown in Table V.

Intraluminal multiplication of organisms was observed with each strain. There was no significant difference in degree of multiplication among strains and regardless of

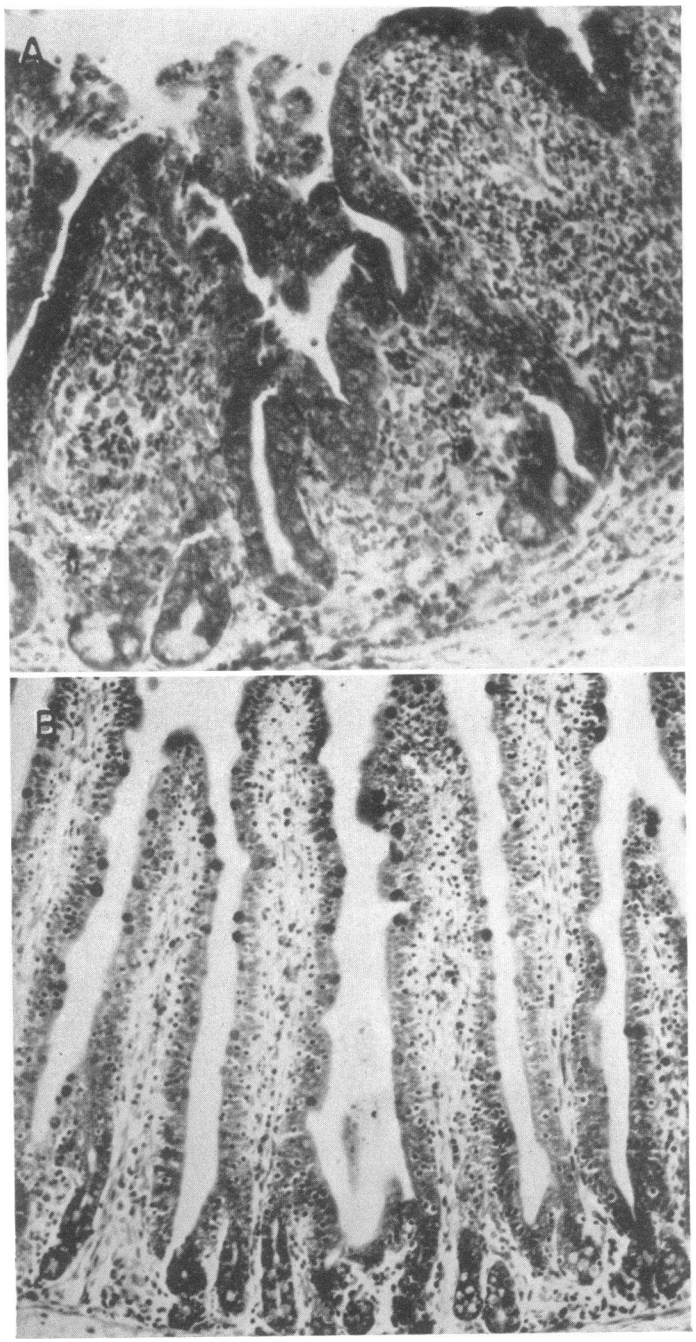

FIGURE 5 Ileal mucosa 18 h after inoculation. (*A*) Strain TML. Villi are swollen, blunted and densely infiltrated with PMN's. Villus epithelium is disordered, hypercellular, and low columnar to cuboidal (Giemsa × 240). (*B*) Strain M206. Normal mucosa except for slight increase in mononuclear cells in lamina propria and a few PMN's at base of villi and in villus cores (Giemsa × 130).

the number inoculated, the total number of organisms recovered per loop at 18 h was the same, i.e. 10^9–10^{10} total organisms.

Effect of cell-free materials on rabbit ileal loop. Both cell-free culture supernatants and whole bacterial cell lysates failed to induce fluid secretion. No morphologic abnormalities of the intestinal mucosa were noted in any of the loops microscopically examined. In addition, neither the sterile, concentrated ileal fluid produced in vivo in response to live TML organisms nor the Van Heyningen extract elicited fluid secretion or mucosal damage. In all, 13 cell-free preparations were tested in 62 rabbits.

Additional studies

The possibility that intramucosal PMN's, containing physiologically active and inflammatory substances (26), might be essential to the pathogenesis of fluid secretion induced by invasive pathogens was assessed by examining the effect of rabbit PMN's on the intestinal mucosa of the ligated rabbit ileum. PMN's were obtained by the technique of intraperitoneal injection of 1% glycogen and paracentesis 8 h later (27). PMN's so obtained were washed in isotonic saline, disrupted by sonication and suspended in isotonic saline. 1 ml of these preparations, containing approximately 10^8 PMN's were inoculated intraluminally and 0.2 ml, also containing 10^8 PMN's inoculated intramurally into the ligated ileal loop and examined after 18 h. Two separate preparations were so examined. None of eight rabbits inoculated intraluminally or four inoculated intramurally demonstrated fluid secretion.

Since cell-free bacterial preparations did not evoke fluid secretion, an attempt was made to detect an intramucosal factor responsible for fluid secretion. Sterile aqueous extracts of TML-infected ileal mucosa were prepared as follows: ligated ileal loops were inoculated

TABLE II

*Peripheral Dissemination of Salmonellae from Rabbit Ileal Loop**

Strain	Time	No. examined	No. positive cultures		
			Heart	Liver	Spleen
	h				
W118	3	5	0	1	1
	5	5	0	0	1
	7	5	0	1	1
	12	5	0	2	2
	18	8	2	7	7
TML	3	5	1	1	1
	5	5	0	0	0
	7	11	1	3	6
	12	5	1	1	1
	18	11	3	11	9
M206	3	6	0	4	2
	5	—	—	—	—
	7	10	0	0	0
	12	6	0	1	0
	18	8	0	0	0
SL 1027	18	14	5	11	11
LT-7	18	5	0	1	1
PG-41	18	8	0	4	4
9SR[2]	18	9	0	0	0
THAX-1	18	5	0	0	0

* 10^9 organisms inoculated in each instance.

TABLE III

*Ileal Fluid Secretion Induced by Various Strains of Salmonella typhimurium**

Strain	Time (hours)				
	3	5	7	12	18
W118	0.27±0.04 (5)	0.19±0.04 (5)	0.36±0.11 (5)	0.65±0.17 (5)	1.36±0.23 (8)
TML	0.27±0.06 (5)	0.19±0.06 (5)	0.31±0.04 (11)	1.05±0.22 (5)	1.48±0.18 (11)
M206	0.11±0.04 (6)	—	0.26±0.05 (10)	0.26±0.11 (6)	1.67±0.18 (8)
Control‡	0.014±0.007 (9)	—	0.01±0.005 (9)	—	0.003±0.002 (28)

* Expressed as mean ±1 SE loop volume (ml): length (cm) ratio. 10^9 organisms inoculated in each instance. Numbers in parentheses represent number of animals studied.
‡ 1 ml of BHI broth inoculated. Fluid secretion significantly greater than control, $P < 0.01$, for each organism at 3, 7, and 18 h.

TABLE IV

*Effect of Number of Organisms on Ileal Fluid Secretion Induced by Salmonella typhimurium**

Strain	Number of organisms				
	10^2	10^4	10^5	10^6	10^9
W118	0.37±0.35 (5)	0.58±0.32 (5)	1.31±0.35 (5)	1.21±0.46 (4)	1.36±0.23 (8)
TML	0.27±0.10 (5)	0.70±0.21 (6)	0.93±0.34 (4)	1.45±0.42 (4)	1.48±0.18 (11)
M206	0.57±0.16 (4)	0.49±0.31 (4)	0.93±0.30 (6)	1.55±0.25 (5)	1.67±0.18 (8)

* See legend to Table III. Time interval is 18 h.

with 10^9 organisms and 18 h later the infected mucosa was homogenized in ice cold isotonic phosphate-buffered saline, centrifuged and the supernatant sterilized by filtration. 2 ml of the extract was inoculated into ligated rabbit ileal loops and the loops examined 18 h later. Four separate extracts were tested in each of two rabbits and none of the eight ligated loops demonstrated fluid production.

DISCUSSION

Little is known of the mechanisms whereby invasive bacteria evoke fluid exsorption by the small intestine. *Salmonella typhimurium,* one of many different enteric pathogens of this type, was chosen as the model for our study. Representative strains, differing in their virulence and behavior as based on the criteria of mortality in the mouse i.p. test, mortality when fed to the starved-opiated guinea pig, and in ability to cause diarrhea when fed to Rhesus monkeys, were further investigated in the rabbit ileal loop model. Although this model has been used to study *Vibrio cholerae* (21, 28, 29), *E. coli* (8, 30–35), shigellae (33–37), and *Vibrio parahemolyticus* (33–35), only a single preliminary report exists in which salmonellae were employed in this model (37). In this study, Taylor and Wilkins were unable to reach a firm conclusion regarding the ability of salmonellae to evoke fluid secretion. The ease with which fluid accumulation can be assessed in the rabbit ileal loop model in conjunction with the advantage of telescoping, into hours, the course of salmonellosis provided an opportunity to quantitate some parameters related to this disease. We have found, in the present study, that some strains clearly provoke fluid loss.

TABLE V

[*Summary of Behavior of Various Salmonella typhimurium Strains in the Rabbit Ileal Loop*

Strain	Mucosal invasion	Acute mucosal inflammation	Fluid secretion
W118	+	+	+
TML	+	+	+
M206	+	+	+
SL 1027	+	+	0
LT-7	+	+	0
PG-41	+	+	0
9SR[2]	0	0	0
THAX-1	0	0	0

The steps in the pathogenesis of salmonellosis may be broadly divided into at least three categories, (*a*) ability to invade the gastrointestinal mucosa; (*b*) ability to cause diarrhea; and (*c*) the ability to disseminate from the intestine and multiply and survive within the reticuloendothelial system. This study is concerned only with the first two steps, i.e., mucosal invasion and diarrhea. Although all strains employed in this study possessed the serological and biochemical properties typical of *Salmonella typhimurium,* certain differences in behavior in the various test systems (mouse and guinea pig LD_{50}, Rhesus monkey, and the rabbit ileal loop) were apparent (Tables I and V). The mouse i.p. inoculation test is an extensively employed method for assessing the "virulence" of salmonellae (19); our results demonstrate no correlation between mouse LD_{50} values and the ability of strains to either invade the gastrointestinal mucosa or evoke fluid secretion, a finding also reported with various strains of *Shigella flexneri* (38). Strains M206, SL 1027, LT-7, and PG-41 would be considered significantly less virulent than strains W118 and TML on the basis of the mouse test. However, all six strains invaded the mucosa but only the latter two strains evoked fluid secretion (Table V). Thus the rabbit ileal loop may be an acceptable model for assessing the invasive and fluid-provoking capacity of various *Salmonella typhimurium* strains and more relevant in this regard than either the mouse or guinea pig LD_{50} inoculation tests or the Rhesus monkey models (Table I).

Mucosal invasion, dissemination of salmonellae across the ileal wall, and morphologic reaction. Strains 9SR-2 and Thax-1 did not invade the mucosa and thus did not disseminate across the bowel wall. Strains W118, TML, SL 1027, LT-7, and PG-41 on the other hand, readily invaded the mucosa and were comparable in that the number of salmonellae attached to the epithelial cells and seen within the mucosa appeared similar. Strain M206, however, showed striking differences in that less attachment to and invasion of the epithelium was distinctly evident. Furthermore, unlike the fully invasive strains, the number of organisms within the mucosa did not increase but diminished with time after inoculation. These differences may be a reflection of both the decreased invasive property of M206 and its rapid clearing from the mucosa by phagocytes. The latter is supported by the observations that, unlike "virulent" strains of *Salmonella*

typhimurium as judged by mortality in mice, strain M206 is unable to multiply and survive within macrophages (18, 19).

As one might expect, only those strains that invaded the mucosa disseminated from the ileal loop as evidenced by our ability to culture salmonellae from heart, liver, or spleen (Table II). Although salmonellae were occasionally seen within lacteals, this was unusual and it is uncertain whether these organisms enter the general circulation directly or via the lymphatic system. Strains W118 and TML were cultured with increasing frequency from the liver and spleen with increasing time from inoculation, while the opposite was observed with M206. The inability of strain M206 to survive within macrophages may also account for its rapid disappearance from liver and spleen after 3 h.

As has been shown by Takeuchi (15) and Takeuchi and Sprinz (16) in the starved-opiated guinea pig model, *Salmonella typhimurium* W118 penetrates the brush border of intestinal epithelial cells. In contrast to shigellae (39), salmonellae do not multiply within epithelial cells but pass into the lamina propria, multiply and are engulfed by phagocytes. Although epithelial cells become low columnar or cuboidal and microvilli appear irregular and reduced in height, ulcer formation is infrequent. Invasion of the mucosa results in an intense infiltration with PMN's possibly as a result of the release of chemotactic factors by bacteria (40).

Our findings in the rabbit ileal loop are consistent with these observations but several additional points can be emphasized, however. Salmonellae attach preferentially to villus tips to invade and multiply there; organisms are not seen in crypt areas. Moreover, invasion is not a uniform process; although sheets of salmonellae are seen in the lumen, only few villi (less than 5%) appear invaded. Furthermore, the sites of invasion are initially unaccompanied by visible evidence of cellular damage or by evidence of inflammation. PMN's soon appear and can be seen marginating in submucosal vessels and streaming up the villus core. This suggests that mucosal damage may in part be related to the host PMN reaction.

Fluid secretion. Our finding that some strains of *Salmonella typhimurium* induce fluid exsorption in the rabbit ileal loop parallels observations with strains of *E. coli, V. cholerae,* and shigellae (8, 21, 28–37). However, it is evident that with *V. cholerae* and toxigenic *E. coli,* intraluminally elaborated enterotoxins are adsorbed to the intestinal epithelium to stimulate mucosal adenyl cyclase-cyclic AMP, the result being active fluid secretion (5, 6, 41, 42). Penetration by these organisms into the intestinal mucosa does not occur. In distinct contrast, shigellae species which penetrate the intestinal epithelium also evoke fluid exsorption (1, 23, 35). A single species of shigella, *Shigella dysenteriae* 1, Shiga bacillus, both penetrates the mucosa and elaborates an enterotoxin (7); either of these attributes can independently produce a positive loop (43). The precise mechanism whereby this organism causes intestinal fluid production is still uncertain.

To explain the mechanism of fluid secretion induced by salmonellae, we first considered enterotoxin elaboration as the basis for fluid exsorption. We were unable to demonstrate an enterotoxin using a variety of in vitro and in vivo methods even though one of the strains examined, TML, was isolated from a patient with fulminant watery diarrhea (10, 11). Our inability to demonstrate an enterotoxin or an intramucosal factor responsible for fluid secretion, however, does not eliminate the possibility that salmonellae, after they have invaded the mucosa, liberate a "toxin" that results in fluid secretion. It seems unlikely, however, that a salmonella enterotoxin analgous to that of *V. cholerae, E. coli,* or of *Shigella dysenteriae* 1, contributes to the fluid loss in salmonellosis.

We then examined other possible factors whereby invasive organisms might induce fluid secretion. Extensive mucosal inflammation does not seem to be either a necessary prerequisite or directly responsible for fluid secretion. This conclusion is based on the following observations (*a*) fluid secretion begins before significant morphologic abnormalities are detectable by light microscopy; (*b*) strain M206, which produces minimal histologic mucosal inflammation evokes fluid secretion comparable to that induced by W118 and TML, strains which cause extensive mucosal inflammation; (*c*) strains SL 1027, LT-7, and PG-41 although invading the mucosa and causing an intense acute inflammatory reaction did not induce fluid exsorption; and (*d*) sonicates of rabbit PMN's inoculated either intraluminally or intramurally did not evoke fluid secretion. These data are consistent with the interpretation that PMN's and their myriad of physiologically active and irritating contents are probably not involved in fluid production.

Our morphologic observations permit few conclusions concerning the possible mechanisms of fluid secretion, i.e. active epithelial secretion or passive transudation of fluid. With the fluid provoking stains W118, TML, and M206, fluid secretion occurred prior to any marked morphologic abnormalities, i.e. substantial fluid secretion at 7 h with only rare focal villus necrotic lesions (Figure 1*A* and 1*B*). In fact, fluid secretion was noted with strain M206 with little mucosal abnormality at any time interval (Figure 5*B*). In addition, strains SL 1027, LT-7, and PG-41 did not induce fluid secretion but produced qualitatively similar lesions to the fluid provoking strains W118 and TML. Certain conclusions seem justified, however. Firstly, villus goblet cells probably contribute little to fluid secretion since goblet cell

mucus expulsion was not seen with strain M206. Secondly, although crypt cells have been thought to secrete fluid in the normal (44) and cholera-infected intestine (45), and while crypt hyperplasia and increased mitotic activity was noted with strains W118 and TML, these features were absent with strain M206.

Our data suggest that, as is the case with shigellae (38), invasion of the mucosa by salmonellae may be necessary to the pathogenesis of fluid secretion (Table V). However, this conclusion is based on only eight strains of *Salmonella typhimurium* and must be confirmed with additional strains. However, the observations that strains SL 1027, LT-7, and PG-41 invade the mucosa but do not evoke fluid exsorption suggest that epithelial cell invasion alone is neither solely responsible nor sufficient stimulus for fluid secretion in salmonellosis. Perhaps a bacterial property in addition to ability to invade, i.e., intramucosal elaboration of a "toxin" or of a substance which stimulates the adenyl cyclase-cyclic AMP mechanism, is necessary for fluid secretion. Since comparable numbers of salmonellae were seen within the mucosa with both the non-fluid provoking (SL 1027, LT-7, and PG-41) and fluid provoking strains (W118 and TML), quantitative differences in number of invading organisms probably does not account for the inability of the former strains to evoke fluid secretion. The observations that the number of intramucosal salmonellae M206 were substantially fewer than with any of the above strains while fluid secretion was comparable further supports this argument. Furthermore, dissemination of organisms into the circulation and peripheral organs is probably not a necessary step in fluid secretion by the small intestine. This conclusion is based on the observations that strain SL 1027 (which did not induce fluid secretion) was cultured as frequently from heart, liver, and spleen as were strains W118 and TML (strains which did induce fluid secretion).

In an in vivo perfusion study of rats infected with *Salmonella typhimurium* W118, Powell et al. (9) demonstrated that the ileal mucosa secreted water and electrolytes. The mechanism of this fluid exsorption, however, was uncertain and the observed results were consistent with either active epithelial secretion or with passive transudation of fluid through a damaged mucosa. Our present study does not shed light on the specific mechanisms of fluid production induced by salmonellae. Whether salmonellae act via the adenyl cyclase-cyclic AMP pathway, as do *V. cholerae* and probably toxigenic strains of *E. coli,* must await further study.

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