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Adherence of *Salmonella typhimurium* to Small-Intestinal Enterocytes of the Rat

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The adherence of radiolabeled *Salmonella typhimurium* to freshly isolated enterocytes of rats was studied. The results established that type 1 fimbriated strains adhered in significantly higher numbers than did related nonfimbriated strains. Adherence was inhibited by D-mannose and methyl α-D-mannoside. Results of kinetic studies indicated that adherence was biphasic; the number of bacteria that adhered per enterocyte remained constant for approximately 20 min and then increased rapidly under the assay conditions. The second phase was associated with structural damage to the enterocytes. The addition of chloramphenicol did not prevent the initial attachment of bacteria to enterocytes but did prevent the second phase. Viable and nonviable bacterial cells adhered to enterocytes, but only viable bacteria were destructive. Freshly isolated enterocytes (trypan blue impermeable) and enterocytes stored overnight (trypan blue permeable) were infected by viable *S. typhimurium* in a similar manner, suggesting that metabolic activity of the host cell was of less consequence than metabolic activity of the bacterial cells. A model for the role of mannose-sensitive fimbriae as a virulence factor is proposed.

Salmonella typhimurium is an enteric pathogen of global importance that infects humans of all ages (1). It generally causes a self-limiting gastroenteritis, although bacteremia may occur in children (17). A variety of virulence factors such as adhesins, exoenzymes, enterotoxin, cytotoxin, and endotoxin (6, 7, 16) have been reported; however, the pathogenesis of infection is complex and not well understood.

S. typhimurium is an example of an organism that causes disease by an invasive mechanism (6). Following contact of the organism with the epithelium, there appears to be a degeneration of the microvilli and the apical cytoplasm (25). As the bacterium advances into the cytoplasm, it is enclosed in a membrane-bound vacuole within the epithelial cell. *Salmonella* strains which cause a typhoidlike disease pass through the cells and enter the lamina propria; strains that cause gastroenteritis remain localized in the mucosa (6).

Although *S. typhimurium* has been well characterized with regard to its overall biochemical and genetic properties, the molecular virulence factors which cause pathological changes in the intestinal mucosa have not been rigorously established. Adherence is generally assumed to be a prerequisite for invasion (7), and there is evidence that some enteric pathogens have fimbriae (Fim^+) that mediate binding of bacterial cells to specific receptors on the surface of cells in the intestine or the urinary tract (24). *S. typhimurium* possesses type 1 fimbriae which are referred to as mannose-sensitive (MS) adhesins because they bind to D-mannose-containing receptors on cell surfaces and the binding is inhibited by D-mannose or methyl α-D-mannoside. However, the role of MS fimbriae in the pathogenesis of *S. typhimurium* has been controversial. For example, Dugoid et al. (4) found that fimbriated forms were only slightly better than nonfimbriated forms in causing infection in mice when administered orally. Conversely, Tanaka and Katsube (26) reported that fimbriated *S. typhimurium* strains had significantly smaller infectivity dose values than did nonfimbriated strains following oral administration to mice. Similar conflicting reports have appeared in studies in which in vitro model systems were used to study mucosal invasion. Jones and Richardson (10) reported that both fimbriated and nonfimbriated strains possess a nonfimbrial mannose-resistant (MR) adhesin which is responsible for the adherence of *S. typhimurium* to HeLa cells. Tavendale et al. (27), however, found that adhesion of *S. typhimurium* to HEp-2 and HeLa cells is dependent on the MS adhesin; the MR adhesin had no significant effect on promoting adhesion of the bacterial cells to either HEp-2 or HeLa cells. Furthermore, they found that the MR adhesin was apparently a diffusible product that easily detached from the bacteria and thus was unlikely to mediate stable bacterial adherence to host cells.

To define the molecular events of *S. typhimurium* adherence to intestinal cells, we initiated studies in which isolated rat enterocytes were used as target cells. All previous in vitro studies, to the best of our knowledge, were performed with cultured epithelial cells as surrogates. The results presented in this report demonstrate that MS fimbriae promote the binding of *S. typhimurium* to rat enterocytes.

MATERIALS AND METHODS

Bacteria. The *S. typhimurium* strains used in these studies are listed in Table 1. Strains S7471N, S7471φF, and LT2 and strains CR8500, S850/PR22, CR6600 (formerly designated TML), AA4000, and AA2202 were kindly provided by P. LoVerde, State University of New York at Buffalo, and G. W. Jones, University of Michigan, respectively.

All stock cultures were grown in 10 ml of Luria broth (19) at 37°C under aerobic and static conditions whereby fimbriae formation is promoted (21). Strains designated phenotypically as Fim^+ agglutinated rat, guinea pig, and human erythrocytes. Hemagglutination was performed by mixing

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TABLE 1. *S. typhimurium* strains used in this study

Strain	Relevant characteristics	Fimbriated phenotype	Reference
S7471N	FIRN biotype[a]	−	18
S7471φF	Transductant of S7471N	+	18
LT2	Wild type	+	18
CR8500	FIRN biotype	−	10
S850/PR22	Transductant of CR8500	+	10
CR6600	Wild type	+	10
LT2(Δ712)	Cryptic plasmid pSLT; Δhis DCBHAFIE712	+	11
AA2202	LT2(Δ712) cryptic plasmid free	+	11

[a] FIRN, fimbriae negative, inositol negative, and rhamnose negative (2). FIRM strains are permanently nonfimbriated and do not spontaneously give rise to fimbriated mutants.

bacteria and erythrocytes in a ratio of approximately 500 to 1 on a microscopic slide or as a tile test (5) and was evaluated after 3 min. Agglutination was inhibited by D-mannose or methyl α-D-mannoside, indicating the presence of MS fimbriae. Pellicle formation by genotypically Fim^+ strains grown under static conditions was readily observed. Genotypically Fim^- strains grown under similar conditions did not show pellicle formation or agglutination of erythrocytes. Some cultures that we received required at least four consecutive daily transfers to fresh broth before maximum fimbriation was obtained. Stock cultures were preserved at −80°C in Luria broth containing 10% glycerol.

For adherence studies, except as noted, cultures were grown statically for 36 h at 37°C in 10 ml of Luria broth in the presence of [8-^{3}H]adenine (10 μCi/ml). Growth was followed by measuring turbidity with a Klett-Summerson colorimeter (no. 66 filter). After 36 h approximately 175 to 180 Klett units were obtained; this corresponded to approximately 10^{10} CFU. The broth culture was centrifuged (RC-5B; Ivan Sorvall, Inc., Norwalk, Conn.) at 10,000 × *g* for 10 min, and the pellet was washed twice in phosphate-buffered saline (PBS) and finally suspended in the fresh isolation buffer described below. Incorporation of [8-^{3}H]adenine was measured by liquid scintillation counting in a liquid scintillation spectrometer (LS 1800; Beckman Instruments, Inc., Fullerton, Calif.). The usual initial specific activity of the radiolabeled bacteria was approximately 1,000 cpm/1 × 10^6 to 3 × 10^6 bacteria.

Enterocytes. Young adult male rats (Sprague-Dawley; weight, 225 to 250 g) were killed by decapitation, and the small intestine was immediately removed. Enterocytes were isolated by using slight modifications of the procedure described by Toyoda et al. (29). Briefly, 25 cm of the small intestine was excised and trimmed of fat and mesentery. Intestinal segments from the most proximal and the most distal small intestine were processed simultaneously. The segment was longitudinally split open and washed twice by gentle agitation in 5 ml of isolation buffer (see below). The segment was incubated for 2 min on a gyratory (New Brunswick Scientific Co., Inc., Edison, N.J.) shaker at 400 rpm at 37°C. All glassware was siliconized, and all incubations were carried out under an atmosphere of 100% oxygen. The supernatant fluid was discarded, and the intestinal segment was suspended in fresh isolation buffer and incubated for another 10 min with shaking. These procedures were repeated twice; and the supernatant fluids were collected, combined, and filtered through four layers of cheesecloth. The cell suspension was centrifuged for 2 min at 1,500 rpm, and the pellet was washed twice and suspended in isolation buffer to an appropriate density. Isolated enterocytes were suspended in the isolation buffer described below and quantitated microscopically with a hemacytometer. Viability was determined by the trypan blue exclusion test. Over 90% of the enterocytes were viable throughout the experimental manipulations. However, after storage overnight at 4°C, 100% of the enterocytes no longer excluded trypan blue and were considered to be nonviable.

The isolation buffer consisted of 24.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 98 mM NaCl, 6 mM KCl, 2 mM KH_2PO_4, 5 mM sodium pyruvate, 6 mM sodium fumarate, 5 mM sodium glutamate, 11.5 mM glucose, 2 mM glutamine, 0.2% (wt/vol) bovine serum albumin, 2% amino acid mixture, and 1% (vol/vol) essential vitamin mixture. The pH of the solution was adjusted to 7.4. All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.), except for amino acid mixture (GIBCO Laboratories, Grand Island, N.Y.) and the vitamin mixture (M.A. Bioproducts, Walkersville, Md.).

Binding assay. Freshly prepared enterocytes and bacteria were used in the binding assay. The suspension of radiolabeled bacteria (0.5 ml) containing approximately 10^7 to 10^9 CFU was incubated in a plastic tube with 0.5 ml of a suspension of enterocytes at 37°C for 30 min on a slowly rotating wheel. In each experiment, a ratio of 500 bacteria per enterocyte was used. Bacteria bound to enterocytes were separated from free bacteria by filtration through a polycarbonate membrane filter (5 μm pore size; Nuclepore Corp., Pleasanton, Calif.). The incubation tube was rinsed twice with PBS, and the washings were filtered through the membrane. The membrane was washed four more times with 5 ml of PBS and transferred to a scintillation vial, and 5.0 ml of scintillation fluid (Liquiscint; National Diagnostics) was added. The sample was counted in a scintillation spectrometer (LS 1800; Beckman). All assays were run in triplicate, and the results were corrected for nonspecific bacterial binding to the membrane filter. This was determined by including control tubes which were incubated in the absence of enterocytes. Less than 0.3% of the bacterial cells present in the assay was retained on the filter. The filtration procedure effectively separated enterocyte-bound bacteria from free bacteria. Vortexing of the assay mixture for 30 s at the end of the incubation period did not significantly reduce the number of bacteria that bound to enterocytes. It should be noted that the initial ratio of bacteria per enterocyte present in the assay system was found to be important in the adherence studies. At bacterial cell numbers of 10^4 or greater per enterocyte, high numbers of fimbriated as well as nonfimbriated bacteria appeared to adhere to enterocytes. However, these results were misleading since microscopic examination revealed that bacterial coaggregation was occurring and would be a complicating factor in the interpretation of experimental results. At lower bacterial cell numbers, between 100 and 1,000 bacterial cells per enterocyte, no coaggregation was observed microscopically; and subsequently, we routinely used 500 bacterial cells per enterocyte in the assay system.

The binding results were expressed as the number of bacteria bound per enterocyte. This value was calculated from the specific activity of the labeled bacterial cells used in the assay. Since bacterial growth could greatly affect the initial specific activity, we determined the increase in bacterial cells that occurred during the incubation period. This was performed for each strain used in the study. Bacterial growth was measured by determining total viable counts in

the presence and absence of enterocytes. In the absence of enterocytes, growth could also be followed by measuring the A_{550} in a spectrophotometer (Ultraspec II; LKB Instruments, Inc., Rockville, Md.). No difference in growth kinetics was observed in the presence or absence of enterocytes. The relative increase in growth for strain S7471ϕF and S7471N during the assay period is seen in Fig. 2 (inset). The relative increase in growth was identical whether growth was determined by measuring viable counts or by measuring the A_{550}. All strains grew slightly and exhibited similar growth kinetics. After 120 min of incubation, there was an increase in growth from 1.5 to 2.0 times the initial number of cells, depending on the strain that was used. For example, strain S7471ϕF was approximately doubled in cell number (see Fig. 2, inset). The specific activity used to calculate the number of bacteria bound per enterocyte was corrected for the increase in the number of bacterial cells.

Strain S7471ϕF increased 1.10, 1.18, 1.27, 1.56, and 2.0 times the initial number of cells after 10, 20, 30, 60, and 120 min, respectively. Thus, if the initial specificity for S7471ϕF was 1 cpm/1,000 bacterial cells, then, for example, after 30 min this corrected to 1 cpm/1,270 bacteria. Similarly, the specific activity was corrected for each time point in the assay.

Electron microscopy. To examine the bacterial surface structure, bacterial suspensions grown in Luria broth were centrifuged, and the pellets were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. The cells were rinsed and the sample was postfixed in 1% osmium tetroxide. Dehydration was performed by using standard ethanol techniques, and the sample was embedded with resin (Maraglas). Staining was done with uranyl acetate and lead citrate.

A shadow-casting technique was used to examine the fimbriae. Bacterial suspensions in PBS were placed on a Formvar-coated, carbon-stabilized grid and fixed in 3% glutaraldehyde for 30 s. The grid was washed twice with distilled water. The sample was placed in a vacuum evaporator at a 60-deg angle and shadowed with platinum carbon pellet. All specimens were examined in an electron microscope (HS-8; Hitachi).

Statistical analysis. Adherence was expressed as the mean

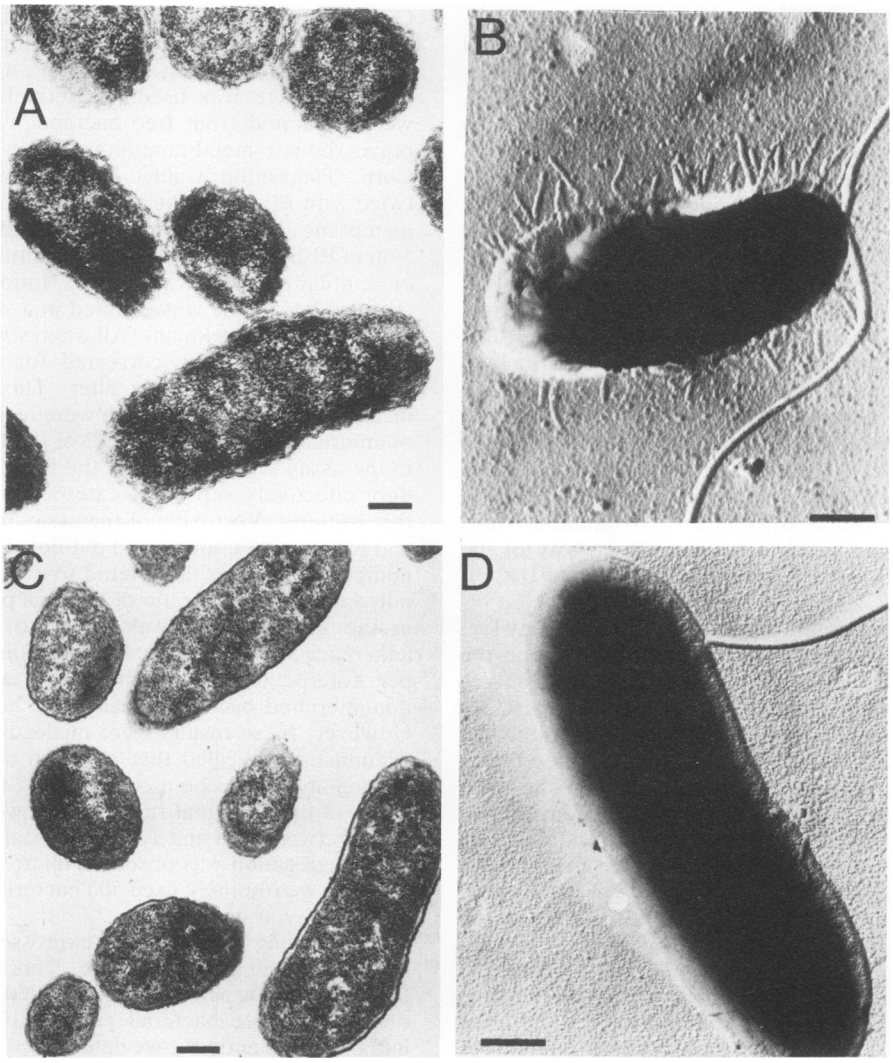

FIG. 1. Electron micrographs of *S. typhimurium* S7471ϕF (Fim^+) (A and B) and S7471N (Fim^-) (C and D). Differences in surface morphology of the Fim^+ and Fim^- strains (panels A and C, respectively) are shown. Fimbriae can be seen on strain S7471ϕF (B) but not on S7471N (D). Note that both strains possess flagella (panels B and D). Magnification, ×19,000 (A and C); ×26,000 (B and D); bars, 0.4 μm.

number of bound bacteria per enterocyte ± the standard deviation. Student's *t*-test was used to test the significance of difference between two means.

RESULTS

Electron microscopy of bacterial cells. The presence or absence of fimbriae on Fim$^+$ (S7471ϕF) and Fim$^-$ (S7471N) bacteria was readily demonstrated by electron microscopy (Fig. 1). In addition, the outer membrane of Fim$^+$ cells appeared to be much more undulating than did its Fim$^-$ counterpart. Similar results were also obtained with CR8500 and its related Fim$^+$ counterpart (S850/PR22).

Adherence of *S. typhimurium* to rat enterocytes. The adherence activities of various Fim$^+$ and Fim$^-$ strains to enterocytes is summarized in Table 2. Fim$^+$ strains attached in much greater numbers than did their related Fim$^-$ counterparts. Type 1 fimbriae are chromosomally encoded (23), and as expected, curing of the cryptic plasmid (strain AA2202) did not reduce adherence. If strain S7471ϕF is grown under conditions whereby type 1 fimbriae are poorly expressed (aerobically in shake culture), only a few bacterial cells adhere to the enterocytes, as do FIRN strains that are permanently Fim$^-$ and do not spontaneously produce Fim$^+$ mutants (2). These results indicate that type 1 fimbriae mediate the attachment of bacteria to enterocytes. No significant difference was found in the number of bacteria that adhered to enterocytes isolated from the distal or proximal segments of the intestine.

The kinetics of adherence is shown in Fig. 2. Adherence was time dependent with a biphasic pattern. A low constant level of attachment of Fim$^+$ bacteria was observed during the first 20 min, followed by a rapid increase in the number of adherent bacteria. Fim$^-$ bacteria showed a constant low level of adherence during the 2 h of the study.

Examination of the incubation mixture by phase-contrast microscopy (Fig. 3) revealed that within the first few minutes the attachment of bacterial cells to enterocytes appeared to be reversible; bacteria could be seen to adhere for several seconds and then move away. After 5 min, however, a small number of bacteria were stably bound to the enterocyte, and the number remained relatively constant during the first 20 min. Bacteria adhered to the brush border surface, as well as to the lateral and basal surfaces, of the enterocyte and did not appear to show any preference for particular regions of the cell surface. After 20 min the number of bacteria adhering to the enterocyte increased rapidly, and after 40 min major structural changes within the enterocyte were noted. These included loss of polarization of the nucleus, vacuolization of the cytoplasm, and formation of cytoplasmic blebs projecting from the cell surface. After 60 min cytoplasmic blebs were released from the enterocyte; this was accompanied by continuous cell destruction. Concomitantly, large numbers of bacteria could be seen to coaggregate and adhere to damaged enterocytes. There was a pronounced increase in bacterial adherence once enterocyte destruction was initiated. Similar morphological alteration of the enterocytes were not observed with Fim$^-$ bacteria even after 2 h of incubation.

TABLE 2. Adherence of *S. typhimurium* to isolated rat enterocytes

Strain	Phenotype	No. of expts	No. of bacteria bound/enterocyte[a]
S7471ϕF	Fim$^+$	14	25.1 ± 13.8
S7471ϕF[b]	Fim$^-$	6	2.7 ± 1.3[c]
S7471N	Fim$^-$	14	5.1 ± 4.0[d]
LT2	Fim$^+$	3	20.3 ± 6.8
CR8500	Fim$^-$	2	9.0 ± 2.5
S850/PR22	Fim$^+$	2	41.8 ± 14.3
CR6600	Fim$^+$	2	30.5 ± 10.4
AA2202	Fim$^+$	2	21.4 ± 5.0
LT2(Δ712)	Fim$^+$	2	18.5 ± 6.6

[a] Mean number of bound bacteria ± standard deviation for the number of experiments indicated. Each experiment was run in triplicate; incubation was performed at 37°C for 30 min.

[b] Grown aerobically in a gyratory shaker (100 rpm; New Brunswick) at 37°C. Cultures were transferred daily for at least four consecutive times before they were labeled with [8-^{3}H]adenine. This procedure rendered the bacteria essentially Fim$^-$ (3). Bacterial cells grown in this manner were unable to agglutinate rat, guinea pig, or human erythrocytes.

[c] Significant difference ($P < 0.001$) of the number of bacterial bound per enterocyte between strains S7471ϕF (Fim$^+$) and S7471ϕF (Fim$^-$).

[d] Significant difference ($P < 0.01$) of the number of bacteria bound per enterocyte between strains S7471ϕF (Fim$^+$) and S7471N.

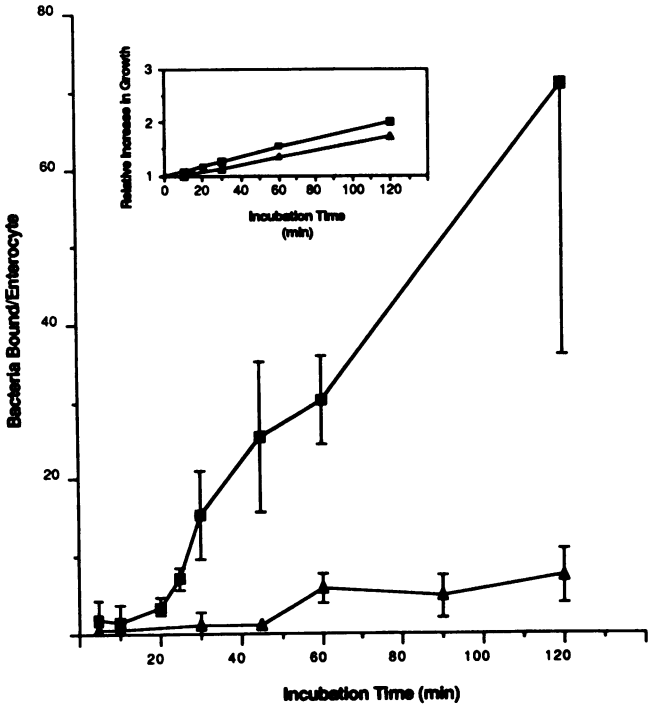

FIG. 2. Effect of incubation time on the adherence of *S. typhimurium* to isolated rat intestinal enterocytes. Symbols: ■, *S. typhimurium* S7471ϕF (fim$^+$); ▲, *S. typhimurium* S7471N (fim$^-$). The values represent the mean number of bound bacteria ± standard deviation after nine experiments with Fim$^+$ bacteria and three experiments with Fim$^-$ bacteria. Each experiment was run in triplicate. The inset shows the relative increase in growth for S7471ϕF (■) and S7471N (▲) incubated in adherence assay buffer.

D-Mannose and methyl α-D-mannoside effectively inhibited the adherence of Fim$^+$ bacteria to enterocytes, while L-fucose and D-galactose had no effect (Table 3). No further increase in adherent bacteria was observed even after 2 h of incubation in the presence of D-mannose. Similar inhibitions were observed when D-mannose or methyl α-D-mannoside was added at time zero or 20 min after incubation was initiated.

Influence of incubation temperature and bacterial and enterocyte viability on attachment of *S. typhimurium* to enterocytes. During the first 20 min of the assay, binding of heat-killed *S. typhimurium* to enterocytes was similar to that observed for viable bacteria (Fig. 4A). Also, incubation at

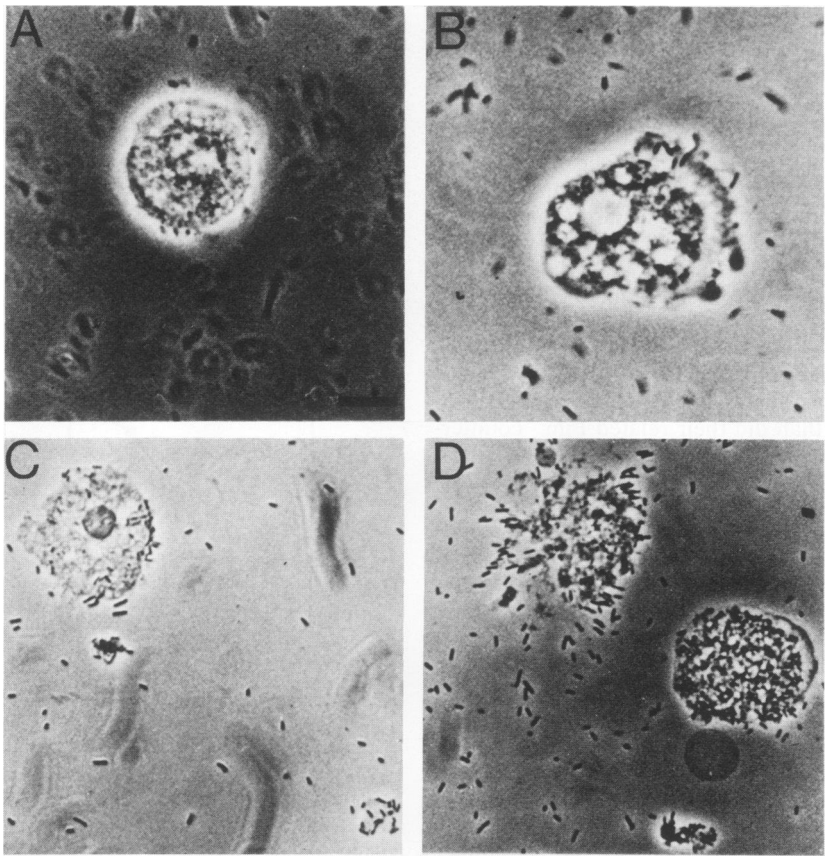

FIG. 3. Phase-contrast micrographs showing the interaction between *S. typhimurium* S7471φF and rat enterocytes. Isolated enterocytes (10^6) were incubated with bacteria (10^9) at 37°C for 5 min (A), 40 min (B), 60 min (C), and 120 min (D) and were examined by phase-contrast microscopy. Magnification, ×760; bar, 5 μm.

4°C did not significantly alter the initial attachment (Fig. 4B). At 4°C, however, the time-dependent increase in bacterial binding was not observed, nor were enterocytes damaged (as judged by phase-contrast microscopy) during the course of the experiment. These observations indicate that after initial binding takes place, ongoing bacterial metabolic processes are required for further infectivity. Thus, it is possible that new proteins are induced as a result of the cell-cell interaction of *S. typhimurium* and enterocytes and that these proteins are essential for virulence. To test whether de novo protein synthesis is required to express the virulent phenotype, we examined the effect of chloramphenicol in the assay (Table 4). As observed with heat treatment, chloramphenicol added at time zero affected only the time-dependent increase of binding but had no effect on the initial attachment of *S. typhimurium* to the enterocyte. When chloramphenicol was added at 10 or 20 min after the incubation was initiated, however, increasing numbers of bacteria adhered, depending on the time of addition of chloramphenicol. Treatment with chloramphenicol did not result in cell death, nor could any difference in fimbria content be detected in untreated and chloramphenicol-treated bacteria (determined by the hemagglutination titer of guinea pig erythrocytes). Thus, we conclude that the bacterium-enterocyte interaction induces the synthesis of new *S. typhimurium* proteins that play a role in virulence. Results of the experiments described above establish that bacterial metabolic activities are required for virulence. To determine whether viable enterocytes are also necessary for the infective process, we compared the kinetics of *S. typhimurium* binding to freshly isolated enterocytes with that of enterocytes that were stored at 4°C overnight. Since stored enterocytes no longer excluded trypan blue, it was assumed that these cells were no longer viable. No difference in the binding pattern was noted (Fig. 4C), suggesting that actively metabolizing host cells are unnecessary for adherence by *S. typhimurium*.

TABLE 3. Effect of various sugars on adherence of Fim$^+$ *S. typhimurium* S7471φF to enterocytes of adult rats

Carbohydrate tested (1 mg/ml)[a]	Adherence[b]	% Inhibition
D-Mannose	1.3 ± 0.6	91
Methyl α-D-mannoside	1.6 ± 0.5	89
L-Fucose	14.7 ± 1.5	0
D-Galactose	13.7 ± 0.5	4
Control (no carbohydrate)	14.2 ± 2.2	

[a] The tested carbohydrate was incubated at time zero with the bacteria and enterocytes, and the adherence assay was carried out under standard conditions.

[b] Adherence is expressed as the mean number ± standard deviation of bound bacteria per enterocyte.

DISCUSSION

To study the molecular mechanism of *S. typhimurium* infection, we used an in vitro model system that consisted of radiolabeled bacterial cells and freshly prepared rat enterocytes. Our results indicate that MS fimbriae played a

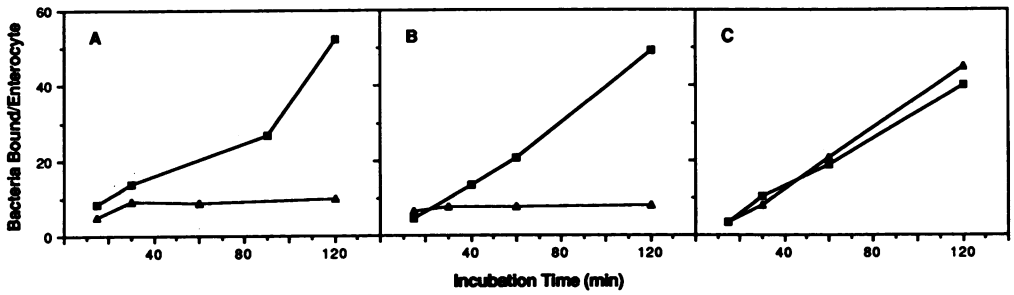

FIG. 4. Effect of various treatments and incubation temperature on adherence of *S. typhimurium* to rat enterocytes. *S. typhimurium* S7471φF (Fim$^+$) was used throughout. (A) Effect of heat treatment of bacteria. Symbols: ■, untreated bacteria; ▲, bacterial cells heated at 60°C for 20 min. (B) Effect of incubation temperature. Symbols: ■, incubated at 37°C; ▲, incubated at 4°C. (C) Effect of stored enterocytes on adherence. Symbols: ▲, fresh enterocytes; ■, enterocytes stored overnight. The values represent the mean number of bound bacteria per enterocyte for two experiments. Each experiment was run in triplicate.

primary role in the attachment of *S. typhimurium* to enterocytes. Thus, our results are in complete agreement with the results of experiments reported by Tavendale et al. (27) on the adherence of *S. typhimurium* to HEp-2 and HeLa cells. Our results differed from those reported by Jones and Richardson (10), who concluded that the MS fimbriae does not play a role in adherence of *S. typhimurium* to HeLa cells. However, as discussed by Tavendale et al. (27), it appears likely that they may have used bacteria which contained few MS fimbriae. We did not detect any difference in adherence to enterocytes isolated from either proximal or distal small intestine, nor was adherence limited to the brush border since bacteria could be seen to adhere to all surfaces of the enterocyte. Of interest in this regard is the study by Knutton et al. (15), in which they indicated that type 1 fimbriae of enterotoxigenic *Escherichia coli* promote adhesion to basolateral rather than brush border surfaces.

Internalization of *S. typhimurium* by nonphagocytic cells has been generally assumed to occur by an energy-requiring endocytic mechanism (14). Kihlstrom and Nilsson (14) demonstrated that invasion of HeLa cells is inhibited by iodoacetate or by cytochalasin B. Also, ultrastructural studies have provided evidence for an endocytic process in that internalized *S. typhimurium* was seen as being enclosed in membrane-bound vacuoles (13). Hale et al. (9) reached similar conclusions regarding internalization of *Shigella* spp. by Henle cells. In either case, viable bacterial cells are required for the infection process (8, 12). In this report we have shown that both viable and nonviable bacterial cells attach to enterocytes but that only viable bacteria are destructive. Because of the fragility of *S. typhimurium*-infected enterocytes, it was not possible to carry out studies that would have differentiated adherence from internalization. Results of our studies do suggest, however, that under the conditions of assay, the infectious process is primarily dependent on bacterial metabolic activity and that metabolic activity of the host cell is of less consequence. It should be noted that viability of enterocytes was determined by trypan blue exclusion, and we have not ruled out the possibility that these cells could still carry out physiological functions that may contribute to the infection process.

TABLE 4. Effect of chloramphenicol on adherence of *S. typhimurium* S7471φF to rat enterocytes

Chloramphenicol addition	No. of bacteria bound/enterocyte after[a]:	
	30 min	120 min
At time zero (μg/ml)		
0	21.3 ± 0.8	119.3 ± 31.3
25	19.4 ± 4.5	43.3 ± 12.2[b]
50	21.7 ± 2.2	31.0 ± 9.9[c]
125	18.2 ± 1.6	26.6 ± 7.5[c]
25 μg/ml at time (min)		
0	6.8 ± 1.6	15.4 ± 3.3[c]
10	5.1 ± 2.7	23.6 ± 3.5[c]
20	12.1 ± 2.4	35.0 ± 9.2
Control[d]	11.2 ± 1.4	51.0 ± 3.6

[a] Mean number of bound bacteria per enterocyte ± standard deviation.
[b] Significant difference ($P < 0.01$) compared with control.
[c] Significant difference ($P < 0.001$) compared with control.
[d] For the control, no chloramphenicol was added.

Results of kinetic studies demonstrated that under the conditions of the assay, adherence of *S. typhimurium* to enterocytes appears to be biphasic. During the first phase, the number of adherent bacteria remained relatively constant for about 20 min. This apparent lag period was followed by a second phase, during which there was a rapid increase in the number of bacterial cells associated with the enterocytes. Destruction of the host cell was also observed during this phase. In the initial phase, we noted, in preliminary ultrastructural studies, that there is a degeneration of enterocyte microvilli and that membrane vesicles or blebs are formed (data not shown). Similar observations were reported by Takeuchi (25) but were not confirmed by Kihlstrom and Latkovic (13) in their study of the interaction of *S. typhimurium* with HeLa cells. Degeneration of microvilli is very likely promoted by MS fimbriae that attach to mannose-containing structures on the enterocyte surface. This conclusion is supported by recent work done by Nishihata et al. (20), who observed that concanavalin A, a lectin with specificity directed toward mannose (or glucose) containing oligosaccharides, causes blebing and fusion of microvilli of rat colonic epithelium. Concanavalin A did not induce endocytosis, as it apparently does with cell cultures (20), but did induce increased permeability of rat colonic epithelium to trypan blue. The second phase of *S. typhimurium* colonization of enterocytes may involve the MR adhesin previously described by Jones and Richardson (10), as well as new factors induced by direct bacterial-enterocyte interaction. Chemotactic mechanisms may also play a role in the increased bacterial adherence observed in the second phase. Perers et al. (22) observed an enhanced association of *S. typhimurium* to damaged small-intestinal mucosa of mice compared with morphologically intact mucosa. In addition, Uhlman and Jones (30) reported that *S. typhimurium* swarms

around HeLa cells that have been damaged by exposure to low pH. This resulted in the release of a diffusible chemoattractant from the host cell. It is conceivable that degeneration of microvilli by attachment of MS-specific fimbriae may also release diffusible attractants. These attractants may induce the synthesis of new bacterial chemoreceptors which, in turn, results in swarming toward the target cell and, ultimately, its subsequent destruction.

In view of the information presented above, we now address the question of the apparent paradox that has repeatedly been reported regarding the role of MS fimbriae. Whereas these appendages appear to mediate attachment in vitro, fimbriated strains may be only slightly better than nonfimbriated variants at initiating infections in vivo. Consequently, several workers (7) have concluded that in vitro adhesin studies may not be useful as models to explain in vivo virulence. We propose the following hypothesis as a possible explanation for this apparent enigma. In vitro, *S. typhimurium* attaches to enterocytes by using MS fimbriae, an association which perturbs the microvilli, causing their degeneration and possibly the release of chemoattractants. Enterocytes antagonized in this manner have increased permeability properties. Bacterial cells respond to the chemoattractants, swarm to the target cell, associate with it, and cause its eventual destruction through as yet undefined factors. In this scheme, we envisage the MS fimbria not as an organelle whose purpose is to attach bacteria to enterocytes but as an effector of microvilli degeneration. It is this signal that sets the stage for the subsequent attachment and penetration of enterocytes by bacterial cells. If this supposition is correct, then substances other than MS fimbriae that can react with microvilli and induce degeneration may effect a similar chain of events. In vivo, enterocytes are presumably constantly undergoing environmental perturbations. For example, dietary components such as plant lectins have been shown to damage the enterocyte brush border (28). This may simulate the action of MS fimbriae in vivo and result in the release of chemoattractants as well as enterocytes with increased permeability properties. *S. typhimurium*, whether Fim^- or Fim^+, might be expected to respond similarly to chemoattractants and show equivalent pathogenicity in animal studies.

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