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Shigella Infection of Henle Intestinal Epithelial Cells: Role of the Host Cell

THOMAS L. HALE,† RANDAL E. MORRIS, AND PETER F. BONVENTRE*
Department of Microbiology, University of Cincinnati Medical Center, Cincinnati, Ohio 45267

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The process of Henle 407 embryonic intestinal epithelial cell infection by *Shigella flexneri* 2a M42-43 was studied in an in vitro model system. The role of the Henle cell was assessed. It was established that entry of *S. flexneri* into cells was suppressed by reagents which inhibit uptake of particles by phagocytic cells. The compounds tested included cytochalasin B, dibutyryl-cyclic adenosine monophosphate, choleragen (*Vibrio cholera* enterotoxin), iodoacetate, and dinitrophenol. Cytochalasin B inhibited infection at concentrations of 1.0 μg/ml or greater. Dibutyryl-cyclic adenosine monophosphate at concentrations of 1 mM and choleragen at 0.1 μg/ml caused significant suppression of infection. Iodoacetate or dinitrophenol, at 0.1 mM concentrations, inhibited internalization of virulent shigellae, and a combination of these compounds inhibited infection at 0.01 mM concentrations. Preincubation of Henle cell monolayers with the combination of iodoacetate and dinitrophenol (0.05 mM) also inhibited infection markedly. The data suggest that infection of epithelial cells by *S. flexneri* in vitro is accomplished by an endocytic process induced by virulent bacteria. The process appears to be similar to uptake of particles by phagocytic cells. Ultrastructural analysis by transmission electron microscopy provided corroborative evidence of phagocytosis of shigellae by Henle cells in that intracellular bacteria were often observed within membrane-limiting vacuoles resembling phagosomes.

The preceding paper established that bacterial metabolic activity is required for initiation of *Shigella flexneri* 2a infection of Henle 407 cells in vitro. It is apparent that the bacterium does not act as an inert particle since nonviable virulent, or viable avirulent, shigellae do not initiate infection of epithelial cells (8). A subsequent set of experiments was designed to delineate the role that the host cell plays in the initiation of *S. flexneri* infection, utilizing the same in vitro system described in the preceding communication.

The rationale for these experiments considered that the host cell might participate in the infection process in either of two ways. The first possibility is that virulent shigellae actually "penetrate" host cells by virtue of one or more virulence factors (enzymes?) which induce membrane damage and subsequent entry of the bacterium into the cytoplasm of the cell. If this mechanism is valid, the host cell would not be required to play an active role in the infection process. It has been proposed that *Toxoplasma gondii* elaborates a penetration-enhancing factor which contributes significantly to infectivity (16), and thus precedent for such a mechanism is established. A second possibility considered more likely was that virulent shigellae gain entry into host cells by an endocytic process. To account for the fact that only virulent strains of *S. flexneri* are invasive, validity of the hypothesis necessitates that virulent forms provide a physical or chemical signal which induces epithelial cells to internalize bacteria by phagocytosis. Utilizing the same logic, it would be assumed that avirulent (noninvasive) bacteria do not induce phagocytosis and thus do not establish infection of epithelial cells. Attempts were made to differentiate between these two possibilities and to establish evidence which would support either of them. As suggested above, the physiological condition of the host cell would not be crucial if shigellae penetrate epithelial cells by breaching the plasma membrane. However, if infection is dependent upon an endocytic process, it is likely that membrane motility and energy requirements would be similar to those of phagocytic cells. Thus, in an attempt to clarify the mechanism of this infectious process, several compounds known to suppress particle engulfment by phagocytic cells were tested for potential effect on infection of Henle 407 cell monolayers by *S. flexneri*.

† Present address: Walter Reed Army Institute of Research, Washington, DC 20012.

The fungal metabolite cytochalasin B suppresses phagocytosis by polymorphonuclear (7, 30) and mononuclear (2, 3) phagocytes. The compound appears to act by disrupting microfilaments which play a role in translocation of the plasma membrane during endocytosis (3). Intracellular levels of cyclic adenosine monophosphate (cAMP) are also known to modify phagocytosis. Exogenous dibutyryl-cAMP, a cAMP analog which traverses the intact cell membrane efficiently, inhibits particle uptake by polymorphs (6, 26) and peritoneal macrophages (29). Phagocytosis is an energy-dependent process linked to glycolysis in polymorphs (21, 23), blood monocytes (5), and peritoneal macrophages (21). Thus, glycolytic inhibitors such as iodoacetate inhibit phagocytosis in all these cell types. Dinitrophenol inhibits phagocytosis by alveolar macrophages since these cells possess an active Krebs cycle (21). The compounds listed above were tested for effects on shigella infection utilizing the standard infection assay described previously (8). In all cases, it was found that infection was reduced or was totally abolished by the phagocytic inhibitors, suggesting that initiation of infection occurs by endocytosis of bacteria. Corroborative evidence for phagocytosis of *S. flexneri* 2a by Henle 407 cells was also obtained by transmission electron microscopy of infected cells. It is concluded that infection by *S. flexneri* 2a requires active host cell participation in the form of phagocytic activity. The endocytic event appears to be induced by factors provided by virulent but not avirulent shigellae.

MATERIALS AND METHODS

Microorganisms. The virulent M42-43 strain of *S. flexneri* 2a has been described previously (8).

Cell culture methods and infection procedure. The Henle 407 human intestinal epithelial cell line (ATCC strain CCL-6) (11) was used as the host cell for these studies. The details of cell culture methods and infection procedure were described earlier (8). When host cells were to be pretreated with *Vibrio cholera* enterotoxin or metabolic inhibitors, these substances were dissolved in Eagle minimal essential medium (alpha MEM; Flow Laboratories, Rockville, Md.) at the indicated concentrations, and 1.0 ml was overlaid on Henle 407 cell monolayers in 35-mm culture dishes. Monolayers were incubated for 3 h at 37°C in 5% CO_2, the MEM was aspirated, and the cells were washed with fresh MEM. The procedure for infection and quantitation was as previously described (8). In experiments involving treatment of host cells before or during infection, data are presented as percentage of control calculated according to the following formula: [mean of percent cells infected (experimental)/mean percent cells infected (control)] × 100 = percentage of control. When indicated, data were subjected to one-way analysis of variance by the Newman-Keuls mean separation test, using a 95% confidence level.

Treatment of Henle 407 cells with cytochalasin B. Cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was prepared as a 1-mg/ml stock solution in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.). This stock solution was diluted in MEM and added to equal volumes of MEM containing approximately 1.0×10^8 colony-forming units of *S. flexneri* 2a M42-43. The bacterial inoculum suspended in graded concentrations of cytochalasin B was then applied to host cell monolayers for the standard 3-h infection period.

Modification of cyclic nucleotide levels in Henle 407 cells. Cell monolayers were exposed to graded concentrations of $N^6,O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid (monosodium salt) (Sigma). Appropriate dilutions of the cAMP analog were freshly prepared in MEM and added to Henle 407 monolayers either before or concomitant with suspensions of virulent shigellae as described above. Experimental controls included addition of shigella suspensions to freshly prepared MEM solutions of 3',5'-cAMP, 5'-5-AMP (sodium salt) (Sigma), or neutralized butyric acid. The bacterial suspensions were immediately added to host cell monolayers, and infection was quantitated as described previously (8). Cholera enterotoxin (Schwarz/Mann, Orangeburg, N.Y.) was dissolved in MEM and incubated with host cell monolayers before or concomitant with *S. flexneri* 2a suspensions.

Treatment of Henle 407 cells with metabolic inhibitors. Iodoacetic acid (Sigma) was dissolved in MEM. Host cell monolayers were preincubated with graded concentrations of the inhibitor where indicated or monolayers were incubated with iodoacetate and virulent shigellae simultaneously. Similar experiments were performed with 2,4-dinitrophenol (Calbiochem, San Diego, Calif.). Dinitrophenol was solubilized in MEM, using a Cole-Parmer ultrasonic cleaner (Cole-Parmer Instrument Co., Chicago, Ill.) to expedite solubilization of the crystals.

Transmission electron microscopy. Processing of samples for transmission electron microscopy was accomplished according to standard procedures. Infected Henle 407 cells were removed from 35-mm plastic culture dishes by incubation in 1.0 ml of 0.2% ethylenediaminetetraacetic acid dissolved in physiological saline. After incubation for 10 min at 37°C, the cells were displaced from the plastic stratum by vigorous pipetting, separated from ethylenediaminetetraacetic acid by low-speed centrifugation, and resuspended in a mixture of 2.5% glutaraldehyde and 2% osmium tetroxide (1:2) prepared in cold cacodylate buffer (12). Fixation was allowed to proceed for 1 h at 4°C. In the specific case of cell cultures exposed to shigellae for 3 h, fixed cells were washed in distilled water and immersed in 1% colloidal thorium suspended in 3% acetic acid for 24 h at 25°C (10). All samples were washed three times in cold saline and postfixed with 0.5% aqueous uranyl acetate in 25°C for 30 min. Samples were dehydrated in ethanol, infiltrated (using acetone as a transition solvent), and embedded in a 1:1 mixture of Epon 812-Araldite 6005

(E. F. Fullam Co., Schenectady, N.Y.). Polymerization was initiated by incubation of infiltrated specimens overnight at 40°C followed by further incubation at 80°C for 24 h. Samples were sectioned with a glass knife, and sections were mounted on copper grids. Grids were stained for 20 min in 2% aqueous uranyl acetate, rinsed extensively in distilled water, counterstained in Reynolds lead citrate for 10 min, and again rinsed in distilled water. Stained preparations were examined with a JEOL 100B electron microscope operated at 100 kV.

RESULTS

Effect of cytochalasin B on infection of Henle 407 cells. If infection of Henle 407 cells by shigellae proceeds in a manner analogous to ingestion of particles by phagocytic cells, the addition of cytochalasin B would be expected to inhibit shigella infection. To test this possibility, *S. flexneri* 2a M42-43 was suspended in MEM containing graded concentrations of cytochalasin B, and the bacterial suspension was immediately added to host cell monolayers. The infection of Henle cells was markedly inhibited by cytochalasin B at a concentration of 1.0 μg/ml (Fig. 1). Higher concentrations virtually abolished infectivity of virulent shigellae.

Effect of 3'5'-cAMP on infection of Henle 407 cells. In the experiments shown in Table 1, equivalent concentrations of dibutyryl-cAMP, 3'5'-cAMP, 5'-AMP, or sodium butyrate were added to suspensions of virulent *S. flexneri* 2a M42-43 in MEM. Bacteria were then added to Henle 407 monolayers and infection was quantitated. Table 1 shows that only dibutyryl-cAMP caused statistically significant inhibition of infection. These data probably reflect solubility of the dibutyryl cyclic nucleotide analog in the lipids of the host cell plasma membrane. cAMP

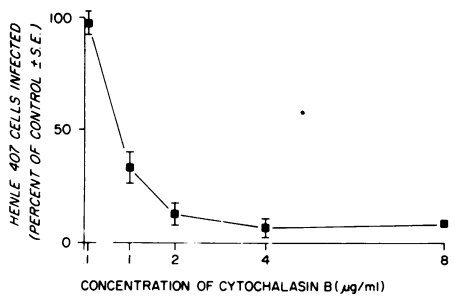

FIG. 1. *Effect of cytochalasin B on susceptibility of Henle 407 cells to infection with S. flexneri 2a M42-43. Cytochalasin B and bacteria were applied concomitantly to Henle 407 cell monolayers, and infection was allowed to proceed for 3 h. Data are expressed as percentage of infection levels relative to controls ± standard error (S.E.) and plotted as a function of cytochalasin B concentration.*

TABLE 1. *Inhibition of Henle 407 cell infection by cyclic nucleotides*

Concn (mM)	Inhibition of infection (%)[a]			
	Dibutyryl-cAMP	3'5'-cAMP	5'-AMP	Butyrate
1.0	76.1 ± 2.0[b]	30.5 ± 6.4	3.4 ± 8.4	13.4 ± 9.8
5.0	74.8 ± 2.0[b]	20.7 ± 3.0	7.1 ± 6.8	
10.0	79.7 ± 3.0[b]			0.0 ± 4.8

[a] All treatments were concomitant with exposure to *S. flexneri* 2a M42-43 for 3 h. Data are expressed as the mean percentage of inhibition ± standard error.
[b] Significant inhibition of infection ($P < 0.05$).

per se does not permeate the intact cell. In addition, it should be noted that butyrate was not an active moiety contributing to inhibition of shigella infection.

In the experiments summarized in Table 1, both the bacterium and the host cell were exposed to dibutyryl-cAMP. Thus, there remained a possibility that inhibition of infection produced by the cyclic nucleotide analog was due to an effect on shigellae as well as the host cell. Therefore, cholera toxin (choleragen), the enterotoxin of *V. cholerae*, was utilized to specifically increase host cell cAMP levels. Cholera enterotoxin binds to the mammalian plasma membrane via ganglioside receptors and activates adenylate cyclase (13). Table 2 shows that infection of Henle 407 monolayers was not altered by simultaneous exposure to choleragen and virulent *S. flexneri* 2a M42-43. However, when the cell cultures were preincubated for 3 h in MEM containing as little as 0.1 μg of choleragen per ml, significant inhibition of infection by virulent shigellae was observed.

Effect of metabolic inhibitors on infection of Henle 407 cells. Experiments were conducted utilizing iodoacetate and dinitrophenol as metabolic probes to define host cell energy requirements of infection. Graded concentrations of each inhibitor were added to MEM containing *S. flexneri* 2a M42-43, and bacterial suspensions were overlaid on Henle 407 cell monolayers. Table 3 reveals a dose-dependent inhibition of infection with either iodoacetate or dinitrophenol. Concentrations of 0.1 mM or greater of either agent caused statistically significant inhibition of infection. Additional experiments were designed to evaluate the effect of metabolic inhibitors in combination. It is seen in Table 4 that a combination of 0.01 mM iodoacetate and 0.01 mM dinitrophenol caused marked inhibition of infection. The data suggest, therefore, that the drugs act synergistically. Although concentrations of iodoacetate and dinitrophenol which suppressed infection in the experiments

TABLE 2. *Inhibition of Henle 407 cell infection by V. cholera enterotoxin*

Expt	Toxin (μg/ml)	Inhibition of infection (%)[a]
1[b]	0.1	10.8 ± 1.3
	1.0	11.5 ± 5.3
	10.0	15.9 ± 15.8
2[c]	0.01	24.2 ± 2.6
	0.1	68.6 ± 5.0[d]
	1.0	58.7 ± 3.2[d]
	10.0	48.1 ± 2.9[d]
	100.0	63.6 ± 7.3[d]

[a] Mean percentage of inhibition ± standard error.
[b] Enterotoxin treatments were concomitant with exposure to *S. flexneri* 2a M42-43 for 3 h.
[c] Henle 407 monolayers were preincubated with enterotoxin for 3 h. Enterotoxin treatment was then continued concomitant with exposure to *S. flexneri* 2a M42-43 for an additional 3-h period.
[d] Significant inhibition of infection ($P < 0.05$).

shown in Tables 3 and 4 were not bacteriostatic for shigellae (data not shown), additional experiments were designed to eliminate exposure of bacteria to metabolic inhibitors during infection. Table 4 shows that preincubation of Henle 407 cells in 0.05 mM combinations of iodoacetate and dinitrophenol for 3 h also resulted in marked suppression of shigella infection. Since pretreated monolayers were washed free of inhibitors before addition of the bacterial inoculum, it can be concluded that the residual inhibition of infection is a consequence of effect on the host cell rather than on the infecting organisms.

Morphological study of infected Henle 407 cells by electron microscopy. A morphological analysis of host cell infection in vitro was performed utilizing transmission electron microscopy. In Henle 407 cells exposed to virulent *S. flexneri* 2a M42-43 for 3 h, some intracellular shigellae were observed lying within membrane-bound vacuoles (Fig. 2A and B) which appear morphologically similar to macrophage phagosomes (19). In addition, partially membrane-bound bacteria and organisms free in the cytoplasm were also observed. It should be noted that a modification in routine staining of glutaraldehyde- and osmium tetroxide-fixed cells was used to prove that membrane-bound bacteria observed in Fig. 2A and B were intracellular. Fixed cells were immersed in colloidal thorium, which adheres to the glycocalyx on the cell surface (10). This technique allows identification of the surface of host cells because the external aspect of the plasma membrane is outlined by thorium particles. No thorium is associated with the bacteria in Fig. 2A and B and, therefore, it is safe to conclude that these bacteria had been engulfed by the host cell before exposure to colloidal thorium.

Infected Henle 407 cells were also processed for electron microscopy after incubation for 18 h in Eagle basal medium with 15% newborn calf serum and 16.5 μg of kanamycin per ml. This procedure allows intracellular multiplication of shigellae in the absence of extracellular bacterial multiplication (8). Figure 3 is a typical electron micrograph showing such nonspecific manifestations of cellular injury as surface blebs, absence of mitochondrial cisternae, appearance of many swollen membrane-bound vesicles, and accumulation of lipid. In addition, the nucleus of the infected cell appears pyknotic with karyolysis of the nucleolus. The ultrastructural alterations observed in infected Henle 407 cells are very similar to those described in cells of the intestinal mucosa of experimentally infected guinea pigs (28) and rhesus monkeys (27).

TABLE 3. *Effect of metabolic inhibitors on infection of Henle 407 cells*

Inhibitor	Concn[a] (mM)	Inhibition of infection (%)[b]
Iodoacetate	0.01	1.2 ± 6.2
	0.1	63.9 ± 5.4[c]
	0.2	94.4 ± 2.9[c]
2,4-Dinitrophenol	0.001	17.9 ± 6.1
	0.01	25.5 ± 3.7
	0.1	67.8 ± 6.9[c]
	1.0	82.6 ± 2.8[c]
	2.0	93.1 ± 0.8[c]

[a] Inhibitor present at concentration indicated during 3-h infection period. Bacterial multiplication was not impaired by iodoacetate or dinitrophenol at these concentrations.
[b] Mean percentage of inhibition ± standard error.
[c] Significant inhibition of infection ($P < 0.05$).

TABLE 4. *Synergistic action of metabolic inhibitors in suppression of Henle 407 cell infection with S. flexneri 2a*

Iodoacetate and 2,4-dinitrophenol[a] (mM)	Preincubation with inhibitors (3 h)	Inhibitor treatment during infection (3 h)	Inhibition of infection (%)[b]
0.001	—[c]	+	3.5 ± 2.6
0.01	—	+	61.0 ± 11.9[d]
0.1	—	+	98.5 ± 0.5[d]
0.02	+	—	7.5 ± 4.7
0.05	+	—	87.1 ± 3.9[d]
0.1	+	—	87.6 ± 1.8[d]

[a] Each inhibitor used at concentration indicated. Bacterial multiplication was not impaired by these concentrations of iodoacetate and dinitrophenol.
[b] Mean percent inhibition ± standard error.
[c] —, Not done.
[d] Significant inhibition of infection ($P < 0.05$).

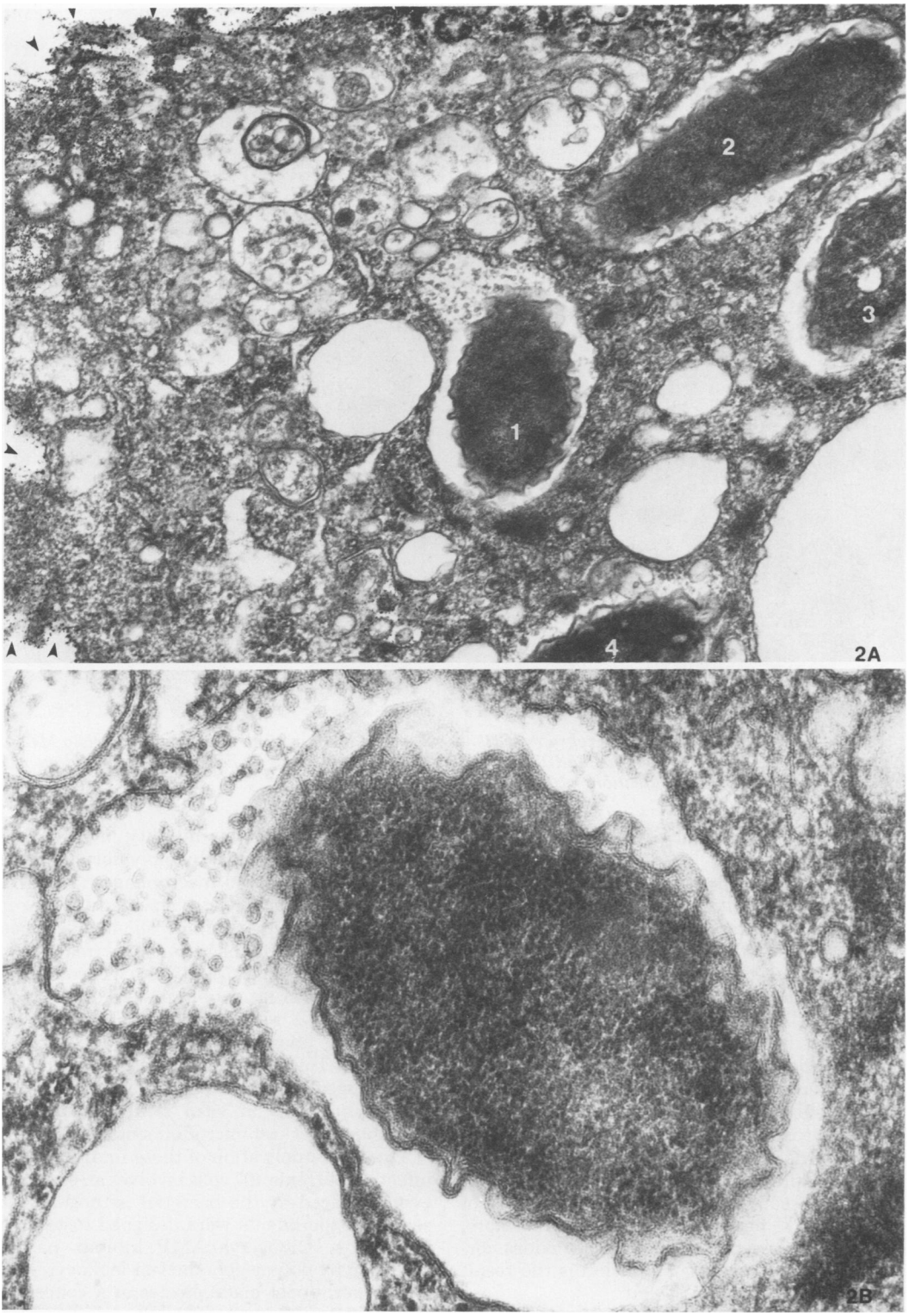

FIG. 2. *(A) Transmission electron micrograph of Henle 407 cell infected with S. flexneri 2a M42-43. Two bacteria are completely membrane bound (1 and 2). Two others appear to be free in the cytoplasm (3 and 4). Note thorotrast marking exterior of the cell (arrows). Sample was processed 3 h after infection. ×24,000. (B) Transmission electron micrograph of the bacterium seen in the center of (A). Note absence of thorotrast associated with phagosome. ×80,000.*

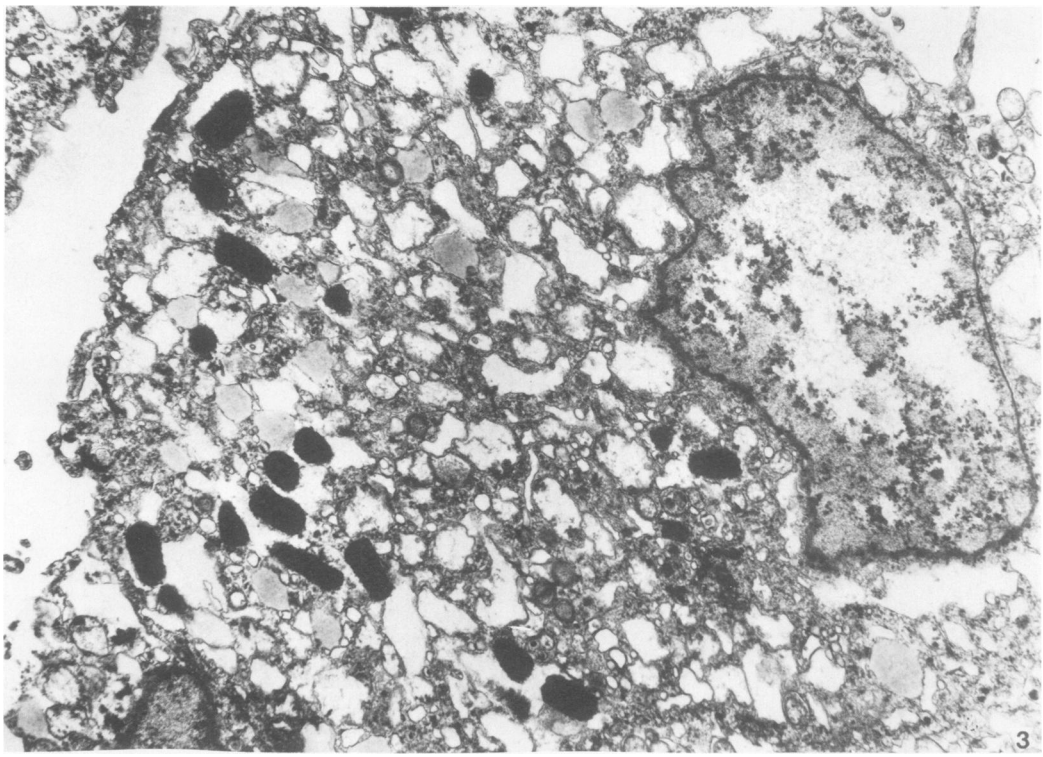

FIG. 3. *Transmission electron micrograph of Henle 407 cell 24 h after infection with S. flexneri 2a M42-43. Note numerous osmophilic bacteria and the advanced state of the host cell degeneration attendant with intracellular bacterial multiplication. ×10,000.*

DISCUSSION

Ogawa et al. (20) studied the interaction of cultured HeLa cells and *S. flexneri* by using phase-contrast time-lapse cinemicrography. They observed that shigellae become associated with the host cell surface and also evoke a marked ruffling of the plasma membrane. Some of the shigellae were enfolded by the ruffles and eventually incorporated into the cells. Thus, morphological studies at the level of light microscopy suggested that infection of mammalian cells by shigellae proceeds by what appears to be endocytosis. Our data support this hypothesis since internalization of *S. flexneri* by Henle 407 intestinal epithelial cells is markedly reduced by a variety of compounds which inhibit uptake of particles by phagocytic cells. Furthermore, transmission electron microscopy provides corroborative evidence that infection is the result of an induced endocytic event.

Carter (4) studied the effects of cytochalasin B on mouse fibroblasts and found that motility and ruffling of cell margins were reversibly inhibited. Subsequent reports indicated that the compound also inhibits motility in HeLa cells (22) and phagocytosis by polymorphonuclear leukocytes (7, 30) and macrophages (2, 3). Numerous investigators have reported the disruption of actin polymers of microfilaments in both phagocytic and nonphagocytic cells treated with cytochalasin B (2, 3, 7, 9, 17, 30). At concentrations which disrupt subplasmalemmal microfilaments (i.e., 1 to 3 μg/ml) and suppress cell motility or phagocytosis (2, 3), uptake of *S. flexneri* by Henle 407 cells is also inhibited. This would suggest that infection of epithelial (nonphagocytic) cells in vitro is dependent upon functional host cell microfilaments.

The clear implication of these findings is that infection of Henle 407 cells involves an endocytic event induced by the bacterial pathogen. Subsequent experiments were designed to test this conclusion. Dibutyryl-cAMP inhibits particle ingestion by polymorphonuclear leukocytes and mouse peritoneal macrophages at a concentration of 2.0 mM (6, 26, 29). Therefore, experiments were conducted to test the effect of dibutyryl-cAMP on the susceptibility of Henle 407 cell to shigella infection. It was found that 1.0

mM dibutyryl-cAMP caused statistically significant inhibition of host cell infection. The fact that infection of Henle 407 cells and phagocytosis are both inhibited by similar concentrations of dibutyryl-cAMP is consistent with the concept that an endocytic event rather than penetration is the mechanism by which shigella initiate infection. Experiments utilizing choleragen, which specifically elevates host cell cAMP levels to render a significant proportion of cells refractory to challenge with virulent shigellae, provided additional corroborative evidence.

Since phagocytosis is an energy-dependent process (25) several experiments were done to determine if disruption of carbohydrate metabolism affected infection of Henle 407 cells with *S. flexneri* 2a. It was found that 0.1 mM iodoacetate or dinitrophenol caused significant inhibition of Henle 407 infection. The same concentration of iodoacetate inhibits phagocytosis by polymorphonuclear leukocytes (23), monocytes (5), peritoneal macrophages (21), and alveolar macrophages (21). Preincubation of host cells with the inhibitors also resulted in suppression of infection, showing that the effect was on the host cells rather than on the bacteria.

Electron microscopy provides corroborative evidence that an event analogous to phagocytosis is involved in internalization of shigellae by cultured epithelial cells. Electron micrographs reveal that intracellular shigellae are often found within membrane-bound structures resembling phagosomes. Takeuchi and co-workers (27, 28) have studied experimentally induced shigellosis in monkeys by using electron microscopic techniques. They observed intracellular bacteria in cells of the intestinal mucosa which were partially or totally enclosed within membrane-bound vesicles. In Henle 407 cells exposed to virulent shigellae for 3 h, some intracellular organisms are found free in the cytoplasm, whereas others are located within complete or partial membrane-bound structures. After 18 h of infection, shigellae appear to be randomly oriented in the highly vacuolated cytoplasm of degenerating host cells. Multiplication of shigellae in the intestinal mucosa in vivo results in host cell damage manifested by ultrastructural abnormalities and sloughing of colonic epithelial cells (27, 28). A similar phenomenon occurs in infected epithelial cells in vitro.

In conclusion, we wish to emphasize that the relationship of our experiments conducted in vitro to the actual processes leading to bacillary dysentery in vivo is unclear. However, the correlation of infectivity for cultured cells with virulence in primate hosts (15, 18) suggests that similar processes are involved. Studies involving disruption of host cell energy metabolism and modulation of cyclic nucleotide levels have demonstrated that the cell plays an active role in the initiation of shigella infection in vitro. These findings, considered in the context of experiments with cytochalasin B indicating that normal cell membrane motility is required, furnish evidence that infection of Henle 407 cells in vitro is accomplished by an endocytic process similar to uptake of particles by phagocytic cells. Since epithelial cells are normally nonphagocytic, an inducing stimulus presumably originates from the infectious agent which initiates membrane activity and endocytosis. Although previous experiments have shown that bacterial metabolic activity is required for production of the putative phagocytic signal (8), the nature of the stimulus remains to be elucidated. Apparently the phagocytic signal is provided by virulent but not by avirulent shigellae and thus can be considered a critical attribute of virulence in *Shigella* sp. A more profound understanding of the mechanism involved in induction of phagocytosis by enteric pathogens could yield enormous dividends for modern medicine in the form of new methods and approaches to the prevention and control of enteric infections.

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