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Shigella Infection of Henle Intestinal Epithelial Cells: Role of the Bacterium

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Epithelial cell infection by *Shigella flexneri* 2a was studied in an in vitro model system. Using the Henle 407 human intestinal epithelial cell line as host cells, a standardized experimental protocol which allowed quantitative measurement of infection was developed. Intracellular residence of infecting organisms was confirmed by indirect fluorescent-antibody staining of unfixed and methanol-fixed (Henle 407) cells and by quantitative bacteriological culture of disrupted host cells after infection. The process of shigella entry into cells was evaluated by chemical or physical modulation of the bacterium under controlled experimental conditions. Shigellae were subjected to mild heat, ultraviolet radiation, aminoglycoside antibiotics, and immunoglobulins raised against *S. flexneri* 2a. The data show that heat-stable antigens on the bacterial surface are not solely responsible for infectivity of *S. flexneri* 2a. Furthermore, it was shown that physiological and synthetic functions of shigellae are required for entry into host cells.

An essential feature of bacillary dysentery (shigellosis) in humans is the development of ulcerative lesions in the colonic mucosa; this breach in the integrity of the epithelium allows erythrocytes and inflammatory elements to reach the intestinal lumen. On the basis of results obtained in studies of (i) oral infection of rhesus monkeys and starved guinea pigs, (ii) infection of guinea pig conjunctiva (Sereney test), and (iii) in vitro infection of HeLa cells, it has been established that the critical event in the onset of overt infection is the entry ("penetration") of virulent shigellae into epithelial cells (15, 20). This insight, gained from experiments utilizing laboratory models, has been useful in understanding the pathogenesis of bacillary dysentery in humans. The most useful experimental model developed to date for studying the pathogenesis of shigellosis employs oral infection of simian hosts. However, the complex symbiotic and antagonistic bacterial relationships in the intestinal environment of conventional animals as well as the technical difficulties inherent in manipulation of an intact animal host make this experimental system unsuitable for analysis of infection at the cellular level. For similar reasons the Sereney test and oral infection of starved guinea pigs are useful primarily as qualitative screening tests for virulence of shigella isolates.

Data which define the role of the bacterium in the entry of virulent shigella into a host cell are fragmentary (8, 24-27). The contribution of the host cell in the initiation of infection is unknown. The primary objective of these studies was to assess the roles of the pathogen and host cell by exploiting an in vitro cell culture model. The tissue culture system developed is amenable to precise experimental manipulation and yields data which can be quantitated accurately. An a priori assumption is that shigella infection of an epithelial-like cell in culture is analogous, at least in its fundamental aspects, to infection of an epithelial cell of the colonic mucosa. Such an assumption is supported by the fact that virulent strains of *Shigella* sp. capable of inducing dysentery in humans or other animals can also infect appropriate tissue culture cells; in contrast, avirulent strains do not infect these same cell lines in vitro (15, 19, 20). These observations also suggest that bacterial virulence factors operative in vivo and in vitro are identical.

Infection of cell cultures in vitro is useful since a relatively uniform population of cells can be infected under defined conditions. This allows for selective modification of either the infectious agent or the host cell (20, 24-27) and constitutes the rationale for these studies. This report describes experiments designed to assess the role of *Shigella flexneri* 2a in the infection of Henle 407 cell monolayers. The in vitro assay devised to quantitate shigella infection is validated. Evidence is presented suggesting that metabolic activity on the part of the infecting bacterium is a prerequisite for entry into the host cell. Specific heat-stable surface antigens unique to *S.*

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flexneri 2a are apparently not the sole factor responsible for the initiation of infection. An accompanying paper focuses on host cell participation in the infection process (10).

MATERIALS AND METHODS

Bacterial strains and maintenance of cultures. The virulent M42-43 strain of *S. flexneri* 2a and the avirulent colonial variant 2457 O were used. *S. flexneri* 2a M42-43, which was originally established from a monkey passage of strain 2457 T, causes overt bacillary dysentery when administered per os to humans (16) or to rhesus monkeys (6). This strain also elicits a fatal enteric infection when fed to starved guinea pigs and induces keratoconjunctivitis when inoculated in the conjunctival sac of the guinea pig eye (Sereney test). Strain M42-43 also infects HeLa cells in vitro, a characteristic shared by all virulent strains of *Shigella* (19). The capacity to enter and multiply within epithelial cells in vivo and in vitro is the cardinal attribute of shigella virulence (15). The avirulent 2457 O strain is an opaque colonial variant of the virulent 2457 T strain. This spontaneously arising mutant does not elicit pathological changes in the intestine of orally challenged guinea pigs or monkeys, does not produce ulcerative lesions of the cornea, and does not infect HeLa cells (15). The shigella strains used in the study were kindly provided by S. Formal, Walter Reed Army Institute of Research.

S. flexneri 2a M42-43 was routinely cultured in Luria broth supplemented with 0.2% glucose and 5 mM $CaCl_2$ (2, 26). To maintain virulence, bacteria were deposited under the eyelid of Hartley strain guinea pigs according to the procedure of Machel et al. (17). After 24 h, a sample of the mucopurulent discharge was plated on infusion agar (BBL) and incubated at 37°C. Green-gold, smooth, translucent (T) colonial forms, seen under the microscope by oblique transmitted light (1), were then reinoculated into Luria broth, and the cultures were incubated at 37°C in a reciprocal shaker for 4 to 6 h. Aliquots were frozen and stored at −70°C until used. It was found that prolonged storage of cultures at −70°C resulted in a loss of virulence as measured by infection of cultured cells in vitro. Therefore, passage of bacteria in the guinea pig eye was repeated at least once monthly to maintain virulence. *S. flexneri* 2a 2457 O was grown in Luria broth, stored at −70°C, and subcultured for experiments utilizing the avirulent mutant.

Tissue culture methods and infection procedure. The established Henle 407 human intestinal epithelial cell line (ATCC strain CCL-6) (12) was maintained in Eagle basal medium (GIBCO, Grand Island, N.Y.) with 15% newborn calf serum (GIBCO), 50 U of penicillin G per ml, 100 μg of streptomycin per ml, and 50 μg of amphotericin B per ml (Fungizone; E. R. Squibb & Sons, Princeton, N.J.). Cells were grown routinely in plastic tissue culture flasks in an atmosphere of 5% CO_2. Confluent stock cultures were trypsinized and seeded at a concentration of approximately 2.0×10^5 cells per 35-mm plastic culture dish (Falcon Plastics, Oxnard, Calif.), and the resulting nonconfluent monolayers were incubated for 18 h in 5% CO_2. Approximately 3 h before exposure to shigellae, the culture medium with antibiotics was aspirated and replaced with antibiotic-free medium. At the same time a frozen vial of *S. flexneri* 2a was thawed, and 1.0 ml of the stock culture was inoculated into 50 ml of Luria broth. This subculture was grown with aeration for 3 h at 37°C, washed in physiological saline, and resuspended in Eagle minimal essential medium (MEM) (Flow Laboratories, Rockville, Md.) at a concentration of approximately 1.0×10^8 colony-forming units (CFU)/ml. The bacterial suspension was then diluted 1:2 in MEM. Occasionally the diluting MEM contained added antibiotics or specific shigella antiserum as described in individual experimental protocols. A final bacterial concentration of approximately 5×10^7 CFU was used as the infecting inoculum. To accomplish infection, the nonconfluent monolayers were washed with 1.0 ml of MEM 18 h after seeding, and 1.0 ml of the bacterial suspension was overlaid. After incubation for 3 h at 37°C in 5% CO_2, the extracellular bacteria were aspirated, and the monolayers were washed four times with MEM. Rinsed monolayers were fixed in methanol and stained with Giemsa. Infection of Henle 407 cells by shigellae was quantitated by light microscopy. In each 35-mm culture dish 500 host cells were examined, and cells exhibiting one or more associated bacteria were recorded as infected. The percentage of cells infected in individual culture dishes was calculated according to the following formula: [cells infected/(infected cells + uninfected cells)] × 100 = percentage of cells infected. Experiments were designed to allow averaging of infection levels by using percentages calculated from three or more infected culture dishes for each datum point.

Preparation of antiserum. Eighteen-hour broth cultures of *S. flexneri* 2a M42-43 were washed three times in physiological saline, suspended in saline to a concentration of 1.0×10^9 per ml, and sterilized by heat at 100°C for 2 h. Female, white New Zealand rabbits were vaccinated intravenously according to the following protocol: 0.25 ml on day 1, 0.5 ml on day 8, 1.0 ml at weekly intervals for the next 7 weeks. Animals were bled by cardiac puncture, and the immunoglobulins were purified from pooled sera by ammonium sulfate fractionation. After dialysis against distilled water, the immunoglobulin preparation exhibited a tube agglutination titer of 1:640 against either the virulent M42-43 or the avirulent 2457 O strain. A 1:32 or 1:64 dilution of the antishigella antibody preparation was used in the experiments.

Fluorescent-antibody tests. Immunofluorescent techniques similar to those previously described by Kihlstrom (13) were used to discriminate extracellular from intracellular bacteria. After incubation with a bacterial suspension for 3 h as described in the infection protocol, cell monolayers were washed four times with MEM and incubated with a 1:32 dilution of rabbit antishigella antibody for 20 min at 37°C. Monolayers were either fixed with methanol or processed without fixation. After treatment with rabbit antibody, cells were washed four times in MEM and incubated for 20 min at 37°C with a 1:32 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit 7S immunoglobulin (Miles Laboratories, Inc., Elkhart, Ind.). Cell cultures were rinsed four times in MEM and mounted in glyc-

erol under a glass cover slip. Specimens were examined by epifluorescence microscopy, using a Zeiss photomicroscope III with exciter filter II and a 530-nm barrier filter. A total of 100 Henle cells in each of three monolayers examined were randomly selected and ordered into five categories containing 0, 1 to 2, 3 to 5, 6 to 10, and greater than 10 fluorescent bacteria per host cell, respectively. The percentage of total host cells counted represented by each of the above categories was then calculated.

Quantitative enumeration of shigellae. Infected monolayers of Henle 407 cells were washed four times with MEM and treated with 0.25% trypsin for 15 min at 37°C. A sample of the single cell suspension was enumerated in a Speirs eosinophil counting chamber (Clay Adams, Division of Becton, Dickinson & Co., Parsippany, N.J.); the remaining cells were chilled to 4°C and disrupted in a model DF 101 Raytheon oscillator (Raytheon Manufacturing Co., Waltham, Mass.) for 60 s at a setting of 0.75 A. This procedure disrupted Henle 407 cells without effect on the viability of shigellae. Samples were removed from the homogenate for quantitation of CFU on infusion agar. Bacteria recovered from the homogenate were assumed to represent the sum of cell-adherent extracellular and intracellular bacteria. To eliminate cell-adherent bacteria, monolayers were incubated in 2 ml of Eagle basal medium with 15% newborn calf serum and 16.5 μg of kanamycin sulfate (Kantrex, Bristol Laboratories, Syracuse, N.Y.) per ml for 6 or 24 h. Infection was evaluated by two methods: (i) monolayers were trypsinized, sonically disrupted, and plated to quantitate CFU as described above, and (ii) cell cultures were fixed with methanol and stained with Giemsa for microscopic evaluation. In either case, evidence of bacterial survival or multiplication after incubation of the infected cells with kanamycin was considered to be confirmation of intracellular residence of shigellae.

Treatment of *S. flexneri* with UV radiation or heat. Cultures of *S. flexneri* 2a M42-43 were grown for 3 h in Luria broth and suspended in MEM at a concentration of approximately 5.0×10^7 CFU/ml. Two milliliters of the bacterial suspension was placed in a 60-mm plastic tissue culture dish (Falcon Plastics) forming a shallow fluid layer. The shigellae were exposed to a UV light source (30-W General Electric Germicidal Lamp) at a distance of 60 cm. The irradiated bacteria were then added to Henle 407 monolayers and incubated in the dark for 3 h at 37°C in 5% CO_2.

Heat inactivation of shigellae was accomplished as follows. Several 5.0-ml samples of bacterial suspensions in MEM containing 5.0×10^7 CFU/ml were placed in a 50-ml flask and shaken in a 56°C water bath for 1, 2, or 5 min, after which the bacterial suspension was immediately cooled in an ice bath. These were added to Henle 407 monolayers following the standard infection procedure.

RESULTS

Infection of Henle 407 cells with *S. flexneri* 2a M42-43. Kinetics of infection of Henle 407 cell monolayers under the standardized conditions of the in vitro assay are shown in Fig. 1.

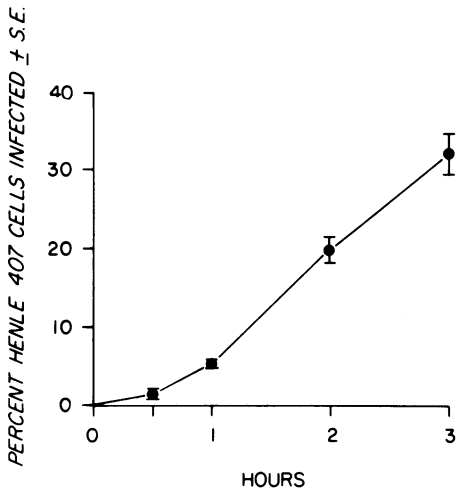

FIG. 1. *Kinetics of Henle 407 cell infection by S. flexneri 2a M42-43. Original inoculum was 1.2×10^8 CFU. After 3 h of incubation in MEM, the number of CFU recovered was 8.7×10^8. The cell density of Henle 407 monolayers was approximately 2×10^5 cells per 35-mm tissue culture dish. Values of infection are based on three cell culture dishes per point.*

After an apparent lag of approximately 30 min, a linear increase in the number of host cells infected was observed during a 3-h period. The initial lag probably reflects the time required for effective contact to be established between shigellae and host cell monolayer as the bacteria settle out of suspension. It is also possible that a metabolic adjustment by the pathogen to altered growth conditions is required before infection is initiated. The data in Fig. 1 are typical for the 3-h infection assay utilized throughout these studies. In this particular experiment, 33% of the cells in the monolayers were infected with shigellae during the 3-h period. Since under laboratory conditions the virulence of *S. flexneri* 2a cultures is unstable (22), it was necessary to devise a system of internal experimental control. To this end, several monolayers were inoculated with the shigella culture to be used in individual experiments. Less than 20% infection of host cells reflected significant loss of virulence, and in those instances where infection fell below this value the experiments were discarded.

Intracellular residence of cell-associated shigellae. The validity of data obtained with the in vitro assay is dependent upon the assumption that cell-associated bacteria viewed in methanol-fixed, Giemsa-stained monolayers are in fact intracellular and not merely adhering to host cell surfaces. Therefore, two techniques were used to distinguish surface-associated from internalized bacteria: (i) indirect immunofluo-

rescence staining of unfixed and methanol-fixed Henle 407 cells, and (ii) quantitative bacteriological culture of disrupted host cells subsequent to incubation with bactericidal concentrations of kanamycin.

The indirect fluorescent-antibody test used to differentiate intracellular and cell surface adherent bacteria is based on the fact that immunoglobulin proteins do not cross the intact plasma membrane (30) but diffuse freely into methanol-fixed cells (29). Figures 2 and 3 are composite results of experiments utilizing immunofluorescence techniques (see Materials and Methods) to evaluate shigella infection of cells in vitro. When Henle 407 monolayers were exposed to a suspension of avirulent *S. flexneri* 2a 2457 O for 3 h, approximately 15% of unfixed host cells exhibited cell-associated fluorescent bacteria (Fig. 2A). However, no cell-associated avirulent shigellae were observed in the methanol-fixed preparations (Fig. 2B). Thus, by the criterion of the test, all cell-associated avirulent shigellae were scored as extracellular. Furthermore, it should be noted that adherent shigellae were totally removed by fixation in methanol. Figure 3A shows that when cell monolayers were exposed to virulent *S. flexneri* 2a M42-43, 37% of the unfixed cells exhibited some degree of association with bacteria. In contrast to avirulent shigella, however, after methanol fixation a significant proportion of host cells was found to be heavily infected (Fig. 3B). Most shigellae associated with methanol-fixed host cells were in fact intracellular since heavily infected cells (e.g., cells with greater than five associated bacteria) were not stained by the fluorescent dye in the unfixed monolayers (Fig. 3A).

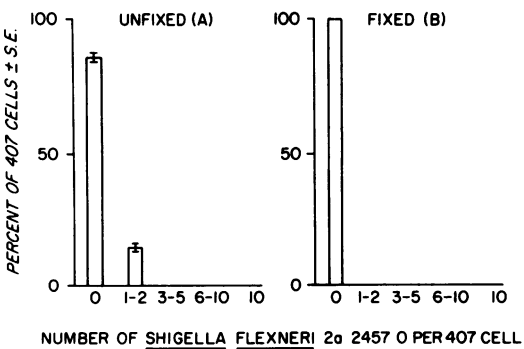

FIG. 2. *Association of avirulent S. flexneri 2a 2457 O with (A) unfixed and (B) methanol-fixed Henle 407 monolayers. Cell-associated bacteria were enumerated by fluorescence microscopy. Data are grouped according to numbers of cell-associated bacteria, and values are presented as the percentage of cells in each group (mean ± standard error).*

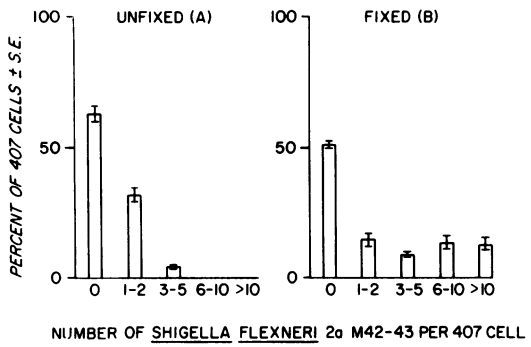

FIG. 3. *Association of virulent S. flexneri 2a M42-43 with (A) unfixed and (B) methanol-fixed Henle 407 monolayers. Cell-associated bacteria were enumerated by fluorescence microscopy. Data are grouped according to the number of cell-associated bacteria, and values are presented as the percentage of cells in each group (mean ± standard error).*

Data verifying the intracellular location of virulent shigellae were also obtained by enumeration of viable bacteria in disrupted Henle 407 cells after incubation with bactericidal levels of kanamycin. Aminoglycoside antibiotics are rapidly bactericidal for *Shigella* species but, because they are not freely diffusible across hydrophobic plasma membranes of mammalian cells (18), are relatively ineffective against intracellular bacteria (23). Kanamycin is bactericidal for *S. flexneri* 2a M42-43 at a concentration of 16.5 μg/ml since a reduction in viability of more than 99.99% is achieved within 3 h (Table 2). Therefore, kanamycin was used to eliminate adherent shigellae in the experiment shown in Fig. 4. After a 3-h exposure to either the virulent M42-43 or the avirulent 2457 O strain, approximately 1.0×10^6 CFU/35-mm culture dish were recovered from disrupted cell monolayers. In the latter instance, however, less than 0.01% of the avirulent shigellae survived a subsequent 6-h exposure to kanamycin, showing that the cell-associated avirulent organisms recovered from the washed cell monolayers were adherent to cell surfaces. In contrast, the total number of recoverable virulent shigellae increased after 6 and 21 h of exposure to kanamycin. Therefore, it can be concluded that the virulent shigellae were protected from kanamycin in the extracellular medium and that these bacteria survived and multiplied within the cytoplasm of the cell. These conclusions were substantiated further by the microscopic evaluation of stained methanol-fixed monolayers (Fig. 4). Approximately 8.0×10^4 cells per Henle 407 culture dish were infected with virulent shigellae, whereas cells exposed to the avirulent strain exhibited no cell-associated bacteria.

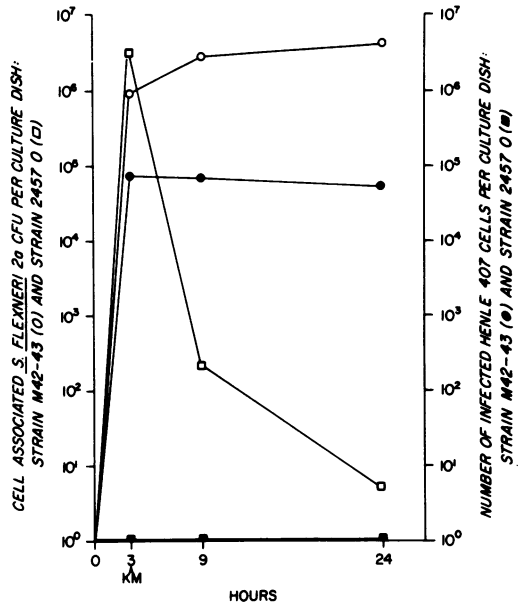

FIG. 4. *Comparison of infection of Henle cells by virulent and avirulent strains of S. flexneri 2a. Kanamycin (KM) (16.5 μg/ml) was added 3 h after initial exposure of Henle 407 cell monolayers to the virulent M42-43* (○) *and the avirulent 2457 O* (□) *strain. Infection was evaluated as either cell-associated CFU per 35-mm culture dish or number of infected cells counted in methanol-fixed, Giemsa-stained monolayers. The total number of cells infected in culture dishes incubated with strain M42-43* (●) *or strain 2457 O* (■) *are values extrapolated from 500 randomly selected cells.*

UV irradiation and heat inactivation of *Shigella*. Table 1 summarizes experiments evaluating infectivity of shigellae pretreated with UV radiation or mild heat. Data showing the relationship of inoculum size (CFU) to percentage of host cells infected in control experiments are included for reference. Exposure of shigellae to UV radiation for 10, 20, or 30 s depressed infectivity. When organisms irradiated for 10 s are compared with normal bacteria, it is apparent that the percentage of cells infected by irradiated bacteria was greater than would be anticipated from comparable untreated inocula measured in terms of CFU (e.g., 2.6×10^5 at 3 h). Microscopic examination of monolayers infected with irradiated shigellae revealed that bacteria infecting the Henle 407 cells were filamentous forms. By viable plate count these filamentous organisms would be considered nonviable since they are incapable of septation and thus unable to form colonies on solid medium. However, bacterial growth and macromolecular synthesis may continue in irradiated organisms (11). We conclude that a large fraction of these metabolically active, nondividing shigellae retained virulence as evidenced by the high levels of infection observed microscopically.

Heating shigellae at 56°C for 2 min resulted in a 3-$\log_{10}$-unit reduction in viability. A minimal infection of Henle 407 cells exposed to heated shigellae occurred. This indicates that loss of viability induced by mild heat treatment also resulted in loss of infectivity. Thus, these data show that infectivity is linked to metabolic activity of shigella. Infectivity may also be linked to heat-sensitive surface proteins modified by heating at 56°C.

Infectivity of kanamycin-treated *S. flexneri*. Experiments were conducted to ascertain if exposure of bacteria to kanamycin modifies infectivity. Virulent *S. flexneri* 2a M42-43 were suspended in MEM containing graded concentrations of kanamycin and applied to Henle 407 monolayers. Table 2 illustrates the effect of kanamycin on viability and infectivity of virulent shigellae. The association of metabolic function with infectivity shown in the previous experi-

TABLE 1. *Infectivity of S. flexneri 2a M42-43 after UV irradiation or mild heat treatment*

Treatment	Inoculum (CFU)[a]	CFU at 3 h	Infection (%)[b]
None (control)	5.6×10^7	1.1×10^9	53.9 ± 0.8
	5.6×10^5	3.3×10^7	4.0 ± 0.3
	5.6×10^3	2.6×10^5	0.1 ± 0.1
	5.6×10^1	1.5×10^3	0.0 ± 0.0
UV radiation			
0 s	5.6×10^7		
10 s	2.7×10^5	1.0×10^5	15.2 ± 2.5
20 s	7.0×10^3	1.0×10^3	1.8 ± 0.6
30 s	1.0×10^1	9.0×10^1	1.6 ± 0.2
Heat (56°C)			
0 min	5.6×10^7		
2 min	5.0×10^4	2.7×10^6	0.1 ± 0.1
5 min	1.5×10^3	8.0×10^3	0.1 ± 0.1
10 min	3.2×10^2	2.6×10^3	0.0 ± 0.0

[a] CFU applied to each Henle 407 monolayer.
[b] Percentage of Henle 407 cells infected after 3 h. Mean values of infected cells in each group ± standard error.

TABLE 2. *Effect of kanamycin on viability and infectivity of S. flexneri 2a M42-43*

Kanamycin (μg/ml)	Inoculum (CFU)[a]	CFU at 3 h	Infection (%)[b]
0	4.9×10^7	1.9×10^9	30.3 ± 0.8
1.65	4.9×10^7	2.1×10^8	8.3 ± 0.1
16.5	4.9×10^7	1.1×10^3	0.3 ± 0.1
165.0	4.9×10^7	1.0×10^1	0.2 ± 0.2

[a] CFU applied to each Henle 407 monolayer. Antibiotic present during the 3-h infection period.
[b] Mean values of infected cells in each group ± standard error.

ment (Table 1) is reinforced by the observation that bactericidal levels of kanamycin quickly ablate the infectivity of *S. flexneri*.

Consequence of antishigella antibody on infectivity. Cultures of virulent shigella were suspended in MEM containing rabbit antibody specific for heat-stable bacterial antigens. The bacterial suspensions were applied to monolayers of Henle 407 cells as in the standard infection assay. Viable plate counts of disrupted monolayers were performed after the 3-h infection period and also after the cell monolayers had been exposed to kanamycin for 6 to 21 h. Cell monolayers infected with virulent shigella not treated with specific antiserum were included in this experiment to establish control infection levels. Figure 5 shows that antibody slightly enhanced rather than diminished the infection of epithelial cells by virulent *S. flexneri* 2a M42-43. Furthermore, antibody did not affect subsequent intracellular multiplication of shigellae. Although antiserum prepared against the M42-43 strain of *S. flexneri* 2a is cross-reactive with the avirulent 2457 O mutant (15), the data show also that specific antishigella immunoglobulin G had no positive effect on the infectivity of the avirulent strain. With or without antibody, strain 2457 O is completely noninfectious in the Henle 407 cell assay. The fact that antibody has no negative effect on the infectivity of *S. flexneri* 2a was also verified by microscopic evaluation of infection. In the presence or absence of antibody, approximately 1.0×10^5 Henle 407 cells per culture dish were infected by strain M42-43. In contrast, infected cells were never observed in Giemsa-stained preparations exposed to the avirulent 2457 O strain.

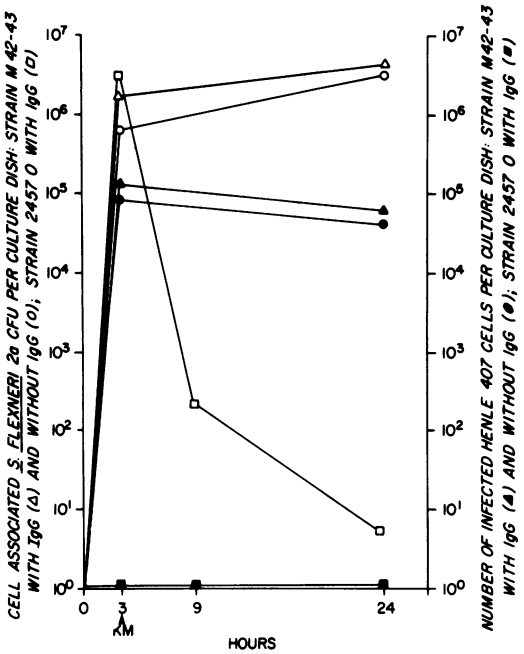

FIG. 5. *Effect of specific antishigella immunoglobulin G (IgG) on infection of Henle 407 cells. Kanamycin (KM), at a concentration of 16.5 μg/ml, was added to the monolayers after 3 h of incubation with virulent (M42-43) shigellae. Data are expressed as cell-associated CFU per 35-mm culture dish in monolayers exposed to shigellae and antishigella IgG (△) or to shigellae alone (○). The number of infected cells per culture dish was determined microscopically in methanol-fixed, Giemsa-stained monolayers infected with shigellae exposed to IgG (▲) or in the absence of IgG (●). Avirulent S. flexneri 2a 2457 O were incubated with Henle 407 cells with or without rabbit IgG raised against the parent M42-43 strain. Infected cells per culture dish exposed to S. flexneri 2a 2457 O, in the presence of IgG (■), were enumerated microscopically, and cell-associated CFU (□) were quantitated before and after addition of KM to eliminate extracellular organisms. Avirulent organisms remained noninfectious in the presence of specific shigella antibody.*

DISCUSSION

Entry of bacteria into the cells of the intestinal mucosa is the key event in the pathogenesis of shigellosis. The capacity to invade intestinal epithelium has become the hallmark of virulent shigellae since this characteristic is an absolute requirement for induction of clinical disease (15). Therefore, ascertaining the mechanism by which *Shigella* spp. gain entry into host cells is of pivotal importance and provided the rationale for initiating these studies.

Tissue cultures as host cells for study of shigella infection have been used with success by Gerber and Watkins (9), LaBrec et al. (15) and Ogawa et al. (20). The present study exploits an infection assay designed to allow an analysis of some important features of epithelial cell infection by *S. flexneri* 2a. Attempts were made to define the role of the bacterium in the initiation of infection. An accompanying report (10) assesses the role of the mammalian host cell in the infection process. The Henle 407 human intestinal epithelial cell line was used as the surrogate host cell, and a protocol allowing quantitative measurement of infection by *S. flexneri* 2a was developed. The assay was validated by confirming the intracellular location of cell-associated shigellae, utilizing immunofluorescence and quantitative bacteriological techniques. To define the contribution of the microbe in this host-parasite relationship, experiments were designed in which *S. flexneri* 2a was modified by physical or chemical means and subsequently tested for infectivity of Henle 407 cells.

It must be assumed that close apposition be-

tween microbe and mammalian cell surfaces is required before infection occurs. Thus, for many years, serious consideration was given to attachment organelles and chemical composition of cell walls as potential determinants of shigella virulence (8). To date no organelles of attachment have been identified; although some strains of shigella possess pili (3), these are not essential since nonpiliated strains of shigella and *Escherichia coli* may invade epithelial cells (21). Gemski et al. (8) have constructed intergeneric hybrids of *S. flexneri* expressing *E. coli* somatic antigens. Some smooth *S. flexneri* hybrids which had acquired *E. coli* factor 25 were found to be virulent, whereas hybrids expressing *E. coli* O-8 antigen were uniformly avirulent. These observations suggest that the chemical composition and structure of the O side chain may represent a factor necessary for bacterial invasion of epithelial cells. However, these workers also isolated three smooth avirulent hybrids expressing the *E. coli* O-25 antigenic specificity, and thus it is apparent that the antigenic configuration of the bacterial surface is not exclusively responsible for infectivity. In addition, qualitative antigenic differences between the virulent 2457 T and the avirulent 2457 O strains of *S. flexneri* 2a have not been detected (15). Our observations using different methodology also fail to incriminate the heat-stable antigenic mosaic of the bacterial surface in the phenotypic expression of virulence. When heated to 56°C for short periods of time, *S. flexneri* 2a M42-43 loses virtually all infectivity for Henle 407 cell monolayers. However, since virulence is undoubtedly multifactorial, a specific somatic antigen complex might be one of several attributes necessary for host cell invasion. Experiments with rabbit antiserum generated with heat-killed vaccine suggest that exposure of specific O-antigen side chains or other regions of bacterial lipopolysaccharide is not required for initiation of infection in vitro. Virulent shigellae were pretreated with antiserum and allowed to infect Henle cell monolayers. When infected monolayers were subsequently fixed with methanol and incubated with fluorescein-conjugated anti-rabbit 7S globulin, uniform fluorescence was seen around the entire perimeter of intracellular organisms (data not shown). This qualitative observation, in addition to the quantitative data presented in Fig. 5, shows that immunoglobulin directed against heat-stable surface antigens does not ablate shigella infection. Indeed, infection of host cell monolayers was slightly enhanced by the presence of antiserum. A report by Ogawa et al. (20) is consistent with these observations since these authors also showed that hyperimmune guinea pig serum failed to reduce infection of HeLa cells by *S. flexneri* 2a.

The high infectivity of antibody-coated shigellae in vitro probably reflects agglutination of the bacterial inoculum promoting efficient contact between bacteria and host cells in the gravity-dependent infection model used. It should be noted, however, that the static conditions characterizing this in vitro system do not obtain in vivo. Thus, agglutination of shigellae in the lumen of the bowel by secretory immunoglobulin A would undoubtedly provide protection. In addition, unlike serum immunoglobulin G, secretory immunoglobulin A may also have an inhibitory effect upon the process of host cell infection per se. This possibility is now being investigated.

The data show also that partial integrity of metabolic function is necessary for infectivity. Primary effects of UV radiation on bacteria are confined chiefly to nucleic acids. Brief exposure to UV light (i.e., 10 s) reduced infectivity of *S. flexneri* markedly but did not abolish it completely. This suggests that heat-sensitive surface antigens are not exclusively responsible for infectivity since these would be preserved after brief exposure to UV. It was observed that UV-treated bacteria retaining ability to invade Henle 407 epithelial cells were filamentous. UV damage of bacterial deoxyribonucleic acids frequently results in loss of the function regulating septation (28), but the organisms retain synthetic integrity as evidenced by continued growth in the absence of cell division. Extensive UV damage as a result of longer exposures (20 s or more) apparently compromises critical synthetic and regulatory functions required for infectivity. The fact that kanamycin quickly aborts the capacity of *S. flexneri* to enter epithelial cells also suggests that loss of infectivity reflects loss of metabolic functions rather than modification of surface structural components. It is interesting to note in this context that UV and short-term gentamicin treatments abolish infectivity of *Salmonella typhimurium* for HeLa cells (14; P. Kaplan, C. E. Benson, and J. S. Gots, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B41, p. 20).

We conclude from these data that virulent shigellae must participate in the infection process in an active fashion, perhaps analogous to rickettsial infection of cultured mouse fibroblasts (31). It should be noted that although bacterial surface properties are not exclusively responsible for infectivity, our data may reflect interrelated virulence factors involving both heat-labile surface components and ongoing metabolic processes. Data presented here and in the following communication (10) suggest that infection of Henle 407 cell monolayers by *S. flexneri* 2a involves induced phagocytosis linked

to the metabolic activity of virulent shigellae. It appears that shigellae in close apposition to the host cell membrane provide a stimulus initiating membrane activity and subsequent phagocytosis of bacteria.

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