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Plaque Formation by Virulent *Shigella flexneri*

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An in vitro tissue culture plaque assay was developed to investigate the intracellular replication and intercellular spread of virulent shigellae. *Shigella* plaques were formed in HeLa cell monolayers in the presence of an agarose overlay containing tissue culture medium and gentamicin, which eliminated extracellular bacterial growth. Microscopically, the plaques were characterized by a central area of dead host cells surrounded by cells infected with shigellae. Cells further away from the plaque center were uninfected. Inclusion of chloramphenicol or nalidixic acid in the overlay completely abolished plaque formation. Plaque formation was completely inhibited when infected monolayers were shifted from 37 to 30°C. Shifting infected monolayers from 30°C, where plaques do not form, to 37°C resulted in the formation of plaques. Cultures of *Shigella boydii*, *Shigella sonnei* (form I), and all six serotypes of *Shigella flexneri* produced plaques. Shigellae isolated from plaques were Sereny test positive, contained a 140-megadalton plasmid, and were gentamicin sensitive. Noninvasive shigellae did not form plaques.

Two essential characteristics of virulent *Shigella flexneri* are the capacity to penetrate the colonic epithelium (1, 9) and the ability to multiply intracellularly (1, 9). Experimental models evaluating the virulent phenotype of *Shigella* spp. include the Sereny test (keratoconjunctivitis shigellosa) (15) and the invasion of cultured mammalian cells (2). Production of keratoconjunctivitis in the guinea pig eye is a result of invasion of the corneal epithelium, replication, and elicitation of a host response. Invasion of cultured mammalian cells by shigellae requires attachment and internalization mechanisms, with the organisms eventually residing in the cytoplasm of the host cell (5, 6, 16, 17).

The spread of shigellae from cell to cell in vivo suggests that a similar process also may occur in cultured cell monolayers. Intercellular spread may occur either by lysis of an infected cell with release of intracellular shigellae or by individual organisms escaping the initially infected cell before lysis and invading contiguous cells without being exposed to the extracellular environment. In the latter case, the initially infected cell may eventually lyse or be killed by resident intracellular shigellae. These interactions suggest that a single, shigellae-infected cell would lead to a progressive infection of neighboring cells, leaving behind an area of dead or dying host cells, i.e., a plaque. In this paper, we report a plaque assay which measures the capacity of invasive shigellae to replicate and spread among cultured mammalian cells after the initial infection by a single bacterium.

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MATERIALS AND METHODS

Bacterial strains and cultural conditions. Pertinent characteristics of the bacterial strains used in this study are shown in Table 1. Cultures were routinely grown overnight at 37°C in Penassay broth and subcultured the next morning in fresh Penassay broth for 4 h. The 4-h cultures were diluted in brain heart infusion broth before inoculation of the HeLa cell monolayers. The Sereny test (15) was used to measure the virulence of the various *Shigella* strains. Tissue culture invasion assays in HeLa cells were performed as previously described (4).

Plaque assay. HeLa cells (Flow Laboratories, Inc., McLean, Va.) to be used in the plaque assay were grown to confluency in 60-mm petri dishes (Costar, Cambridge, Mass.) at 37°C in a humidified atmosphere of 7% CO_2–93% air. Antibiotic-free Eagle minimal essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) containing 5% heat-inactivated fetal bovine serum (FBS; GIBCO Laboratories) was used for the tissue culture medium. Occasionally, HeLa cells grown in the presence of penicillin (100 U/ml) and streptomycin (100 μg/ml) also were used. In preparation for the plaque assay, the monolayers were washed twice with Dulbecco MEM with 4.5 g of glucose (DMEM; GIBCO Laboratories) per liter without antibiotics or serum. After aspirating the second wash, 0.2 ml of the diluted bacterial suspension was added to the monolayer, which was subsequently incubated at 37°C for 90 min. During this adsorption or attachment phase, the plates were rocked every 30 min to assure uniform distribution of the plaque-forming bacteria. Next, an agarose overlay (5 ml) consisting of DMEM, 5% FBS, 20 μg of gentamicin (GIBCO Laboratories) per ml, and 0.5% agarose (SeaKem ME; FMC Corp., Marine Colloids Div., Rockland, Maine) was added to each plate. The plates were incubated at 37°C in a humidified 7% CO_2–93% air atmosphere and examined daily for up to 7 days for plaque formation. For enhanced visualization of the plaques, an optional overlay consisting of DMEM, 0.01% neutral red (GIBCO Laboratories), 20 μg of gentamicin per ml, and 0.5% agarose was added on day 3.

Isolation of plaques. Clearly isolated plaques were aspirated with a sterile Pasteur pipette, placed directly into Penassay broth, and then incubated at 37°C. Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates were inoculated from the broth cultures after several hours

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TABLE 1. Plaque formation by various *Shigella* strains and serotypes

Species	Strain	Sero-type	TCI[a]	Sereny test	Plaque forma-tion	Vir[b] plasmid	Source or reference
S. flexneri	1Z	1b	+	+	+	+	WRAIR[c]
	M25-8	1b	+	+	+	+	14
	M25-8A	1b	−	−	−	−	14
	M25-8A Nal[r] (pWR110)	1b	+	+	+	+	14
	2457 T	2a	+	+	+	+	9
	2457 O	2a	−	−	−	+[d]	9
	J17B	3a	+	+	+	+	14
	Wills	4	+	+	+	+	WRAIR
	M9OT	5	+	+	+	+	14
	M9OT A1	5	−	−	−	+[e]	14
	M9OT A2	5	−	−	−	−	14
	SSU 2407	6	+	+	+	+	WRAIR
S. sonnei	53G form I		+	+	+	+	8
	53G form II		−	−	−	−	8
	212 form I		+	+	+	+	WRAIR
S. boydii	57/61	9	+	+	+	+	WRAIR
E. coli/*Shigella* hybrid	7262-1-10 (pWR110)[f]		+	−	+	+	13

[a] TCI, Tissue culture invasion.
[b] Presence of large-molecular-weight virulence plasmid.
[c] WRAIR, Walter Reed Army Institute of Research collection.
[d] Although the virulence plasmid is present in strain 2457 O, it is not expressed (T. L. Hale, E. Oaks, and S. Formal, manuscript in preparation).
[e] Strain M9OT A1 contains a deletion in the virulence plasmid (14).
[f] This hybrid contains only the 140-megadalton plasmid (pWR110) from *S. flexneri* 5 in an *E. coli* 395-1 background.

or overnight growth. These plaque isolates were serologically confirmed with rabbit antisera specific for group and type antigens of *Shigella* spp.

Intracellular growth of shigellae. HeLa cells growing on eight-chambered slides (Miles Scientific Div., Miles Laboratories, Inc., Naperville, Ill.) were infected with an inoculum (0.2 ml) of *Shigella flexneri* 5 (M9OT) diluted in brain heart infusion broth, after washing the HeLa cells twice with MEM. The infected cultures were placed at 37°C in a humidified, 7% CO_2–93% air atmosphere for 90 min. After the bacterial inoculum was removed, the HeLa cells were washed twice with MEM containing 5% FBS and 20 μg of gentamicin per ml. The infected cultures were finally placed at 37 or 30°C after 0.2 ml of DMEM with 5% FBS and gentamicin (20 μg/ml) was added to each chamber. Slides were stained by the Giemsa method immediately after the 90-min adsorption period (zero time) and at 30-min intervals thereafter for 4 h. The number of shigellae per infected cell and the percentage of cells infected were determined for each time point by light microscopy analysis of the stained slides.

RESULTS

Plaque morphology. Macroscopically, the plaques formed by shigellae have a clear central area surrounded by HeLa cells of the intact monolayer (Fig. 1D). The plaque size was uniform for a given strain, with the diameter reaching approximately 1.0 mm at 3 days postinfection.

Microscopically, the central area of the plaque was devoid of intact host cells (Fig. 1A, B, and C). HeLa cells adjacent to and often two to three cells further removed from the plaque center contained many shigellae. Beyond the infected HeLa cells were uninfected cells (Fig. 1B) representing the intact monolayer.

Characteristics of the adsorption phase. The effect of temperature on the attachment and eventual internalization of shigellae was determined by incubating the shigellae host cell mixture at 4, 25, and 37°C during the adsorption phase of the plaque assay. Shigellae incubated with HeLa cells at 4 and 25°C for 90 min formed less than 1 and 17%, respectively, of the number of plaques formed when incubated at 37°C (Table 2).

Early experiments indicated that the uptake of shigellae by HeLa cells was inefficient. This resulted in a low frequency of plaque formation of about 10^{-4} to 10^{-5} plaques per CFU. One parameter that had a direct effect on the efficiency of forming plaques was the length of the adsorption phase. Time course experiments monitoring the number of plaques formed with increasing adsorption time showed that the plaque titer increased linearly with time. To maintain the viability of the monolayers, 90 min was chosen as a standard adsorption time.

Characteristics of the intracellular growth phase of plaque-forming shigellae. The plaque assay overlay contains the aminoglycoside antibiotic gentamicin, which does not enter the host cell cytoplasm in bactericidal concentrations (7, 10). Extracellular shigellae are killed, however, as attempts to isolate viable shigellae from non-plaque areas of an infected monolayer were uniformly negative. Inclusion of chloramphenicol (5 or 20 μg/ml) or nalidixic acid (1, 5, or 10 μg/ml) in the overlay in addition to gentamicin resulted in complete inhibition of plaque formation (Table 3) due to cessation of intracellular growth.

One factor that had a significant effect on plaque size was the overlay medium. Tissue culture media such as MEM did not permit the formation of large plaques (Table 4), which are found when DMEM is present in the overlay. Compared with MEM, DMEM has a higher concentration of several nutrients, vitamins, and minerals. Therefore, in an attempt to restore the capacity to form large plaques, MEM was supplemented with glucose, pyruvate, or iron. The overlay containing MEM supplemented with a high concentration of glucose permitted plaques of normal size to form, whereas the pyruvate and iron supplements had minimal effects on plaque diameter.

Recently, it was reported that shigellae grown at 30°C had a reduced capacity to enter cultured Henle cells (11). As expected, *S. flexneri* 5 (M9OT) previously grown at 30°C formed fewer plaques (90% reduction) than did organisms grown at 37°C (data not shown). These experiments, in which the adsorption phase was at 37°C, indicate that the temperature of growth somehow alters the capacity of shigellae to enter host cells.

The effect of temperature on plaque formation by shigellae previously grown and also incubated with the host cells at 37°C during the adsorption phase was determined by shifting replicate plaque dishes from 37 to 30°C at 0, 24, 48, and 72 h postinfection (Table 5). The plaque titer and diameter did not change once the infected monolayers were shifted to the lower temperature, although it was possible to isolate viable shigellae from these plaques. Plaque dishes incubated at 30°C from time 0 did not form plaques up to day 6, but upon prolonged incubation (8 days) two plaques with normal morphology were found. Infected monolayers initially placed at 30°C for 1 or 6 days did not contain demonstrable plaques until the monolayers were incubated at 37°C for an additional 48 h. However, in these experiments the number of plaques was reduced as compared with those in dishes initially incubated at 37°C (Table 5).

The lack of plaque formation at 30°C suggested that either

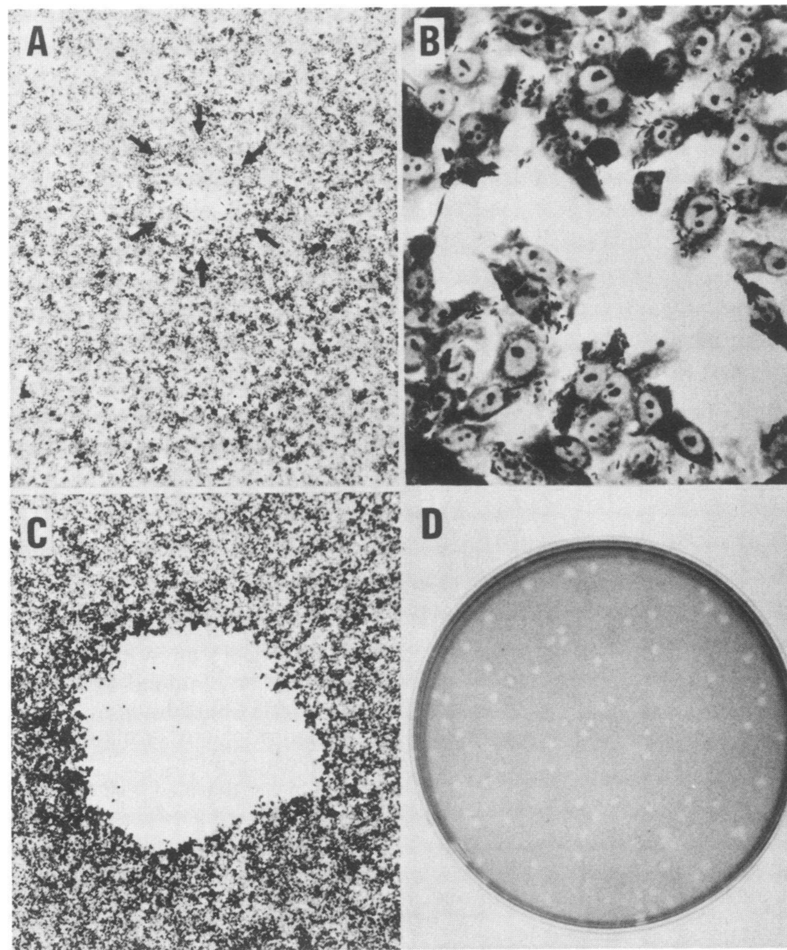

FIG. 1. Plaque formation in HeLa cells by *S. flexneri* 5 (M9OT). Giemsa-stained monolayers demonstrate shigellae plaques at 24 h postinfection ([A] magnification, ×40; arrows point to plaque; [B] magnification, ×160) and at 72 h postinfection ([C] magnification, ×40). Several shigellae plaques in an infected monolayer stained with neutral red at 72 h postinfection are shown in D.

the intracellular shigellae could not multiply at this temperature or that they were unable to penetrate neighboring cells. Examination of infected cells by light microscopy clearly demonstrated that the number of shigellae per infected cell increased at both 30 and 37°C (Fig. 2), indicating that the capacity to replicate was not inhibited at the lower temperature.

Plaque formation by *Shigella* strains and *Escherichia coli*-*Shigella* hybrid strains. All representative virulent strains of the six *S. flexneri* serotypes were able to produce plaques (Table 1). The *S. flexneri* strains tested produced plaques of similar size and morphology in HeLa cells. *Shigella sonnei* and *Shigella boydii* strains also produced plaques. Plaque formation was not demonstrated with *Shigella dysenteriae* due to the high quantity of toxin produced by these organisms which resulted in death of the host cells by 24 h postinfection. Organisms isolated from the plaques were the same serotype as the original inoculum and were Sereny test positive, and all contained the large virulence plasmid. Avirulent strains, which do not produce keratoconjunctivitis in the guinea pig eye and which lack the virulence plasmid, did not form plaques.

TABLE 2. Effect of adsorption temperature on plaque formation by *S. flexneri* 5 (M9OT)[a]

Adsorption temp (°C)	Plaque titer[b]	% of 37°C value
4	$2.0 \times 10^1 \pm 0.9 \times 10^1$	0.1
25	$3.0 \times 10^3 \pm 1.1 \times 10^3$	17.6
37	$1.7 \times 10^4 \pm 0.5 \times 10^4$	100

[a] Bacteria for this experiment were grown at 37°C and placed on the HeLa cells for the adsorption step at the temperatures indicated, and all dishes then were placed at 37°C for plaque formation.

[b] Values represent the mean ± standard deviation of five replica dishes and are expressed as PFU per milliliter.

Previously, it has been shown that the 140-megadalton plasmid of *S. flexneri* can convert a noninvasive *E. coli* into a strain (7262-1-10) which is capable of entering HeLa cells (13). Additional DNA from the chromosome of shigellae was required to construct a fully virulent *E. coli-Shigella* hybrid. In the plaque assay, hybrid strain 7262-1-10 was able to produce plaques, but the *E. coli* parent (395-1) was not. Other *E. coli-Shigella* hybrids (7293-2-2, 7300-1-2, and 7300-1-5) (13), which contain various regions of the *Shigella* chromosome in addition to the *Shigella* plasmid, also formed plaques. The plaques formed by the *E. coli-Shigella* hybrids were smaller than wild-type shigellae plaques and were irregularly shaped. Microscopic examination of Giemsa-stained monolayers infected with the hybrid strains con-

TABLE 3. Effect of antibiotics on plaque formation by *S. flexneri*

Antibiotic[a] (μg/ml)	Effect on plaque formation[b]: *S. flexneri* 5 (M9OT) (Chl[s] Nal[s] Gen[s])[b]	*S. flexneri* 1b M25-8A(pWR110) (Chl[s] Nal[r] Gen[s])[b]
Chloramphenicol		
0	74 ± 9	ND
5	0	ND
20	0	ND
Nalidixic acid		
0	301 ± 24	29 ± 2
1.0	0	30 ± 4
5.0	0	26 ± 2
10.0	0	28 ± 1
50.0	0[c]	0[c]

[a] Antibiotics were present in the agarose overlay in addition to the standard quantity of gentamicin (20 μg/ml).
[b] Resistance (r) or susceptibility (s) to chloramphenicol (Chl), nalidixic acid (Nal), and gentamicin (Gen). Values represent the mean ± standard deviation of five dishes and are expressed as PFU per dish. ND, Not determined.
[c] Nalidixic acid at this concentration was toxic to the HeLa cell monolayers.

firmed that the bacteria were intracellular and were at the site of the plaque.

DISCUSSION

Virulent shigellae characteristically invade the colonic epithelium by a complex process involving recognition of the target tissue, internalization of the shigellae by an endocytic mechanism, escape from the phagocytic vesicle, and subsequent intracellular multiplication. Spread to deeper tissue (lamina propria) or neighboring epithelial cells occurs by an undetermined mechanism. Either the shigellae escape via lysis of the initially infected host cell and then enter a nearby host cell or they bud through the membranes of the initially infected host cell into a neighboring cell.

Two experimental models are widely used to study the invasive properties of shigellae. The Sereny test is an in vivo assay which measures the capacity of shigellae to invade the corneal epithelium of guinea pigs resulting in keratoconjunctivitis. Organisms often are isolated from a positive Sereny test to ensure a virulent culture. The invasion of tissue culture cells also is used as an indicator of virulence. In this assay, mammalian cells previously incubated with shigellae are stained and examined microscopically for the presence or absence of cell-associated bacteria. HeLa cell invasion measures the early stages (attachment and internalization) of shigella-host cell interactions. The Sereny test measures not only attachment and internalization but also intracellular multiplication and spread to neighboring tissue with a subsequent host response.

Plaque formation by shigellae, as described in this paper, measures a complex series of events. A plaque is formed only after attachment, internalization, escape from the phagosomal membrane, intracellular replication in the cytoplasm of the host cell, and subsequent spread to neighboring cells have occurred by the bacteria, leaving behind an area of dead host cells. The high-molecular-weight virulence plasmid of *S. flexneri*, *S. boydii*, and *S. sonnei* (12, 14) is required for plaque formation, as strains lacking this plasmid

TABLE 4. Effect of various media supplements on plaque formation by *S. flexneri*

Media[a]	PFU per dish[b]	Plaque diam (mm) at 72 h[c]
MEM	45 ± 15	Pinpoint
MEM + glucose (4.5 gm/liter)	87 ± 8	0.7 ± 0.3
MEM + pyruvate (110 mg/liter)	47 ± 11	Pinpoint
MEM + $FeCl_3$ (6.7 μg/liter)	46 ± 12	Pinpoint
MEM + glucose + $FeCl_3$	77 ± 16	0.6 ± 0.3
MEM + glucose + $FeCl_3$ + pyruvate	66 ± 12	0.7 ± 0.3
DMEM	84 ± 8	0.8 ± 0.1

[a] These media were present in the agarose overlay which also contains 5% FBS and gentamicin.
[b] Values represent the mean ± standard deviation of five dishes per experiment.
[c] Values represent the mean ± standard deviation; at least 15 plaques were measured per condition.

TABLE 5. Effect of incubation temperature on plaque formation[a]

Incubation conditions	PFU per dish	Plaque diam (mm)[b] on day: 3	5
37°C	304 ± 22[c]	1.0 ± 0.0	1.3 ± 0.1
37°C (24 h) → 30°C	Too small to count	Pinpoint	Pinpoint[d]
37°C (48 h) → 30°C	325 ± 21[c]	0.5 ± 0.1	0.4 ± 0.1[d]
37°C (72 h) → 30°C	277 ± 20[c]	1.0 ± 0.1	1.0 ± 0.1[d]
30°C	0[c,e]		
30°C (24 h) → 37°C	143 ± 29[c]	0.5 ± 0.2	1.1 ± 0.0[d]
30°C (6 days) → 37°C	44 ± 15[f]		

[a] *S. flexneri* 5 (M9OT) was grown and adsorbed at 37°C. Subsequently, the plaque dishes were incubated at the temperatures and times indicated.
[b] Values represent the mean ± standard deviation of 15 plaques measured per condition.
[c] Plaques were counted 72 h from the time of infection. Values represent the mean ± standard deviation of five plaque dishes.
[d] Viable, gentamicin-sensitive shigellae were isolated from plaques on these dishes.
[e] At 8 days postinfection, two plaques (diameter, 1 mm) were found on plaque dishes incubated at only 30°C. Both isolates were gentamicin sensitive.
[f] Number of plaques 48 h after shift up to 37°C.

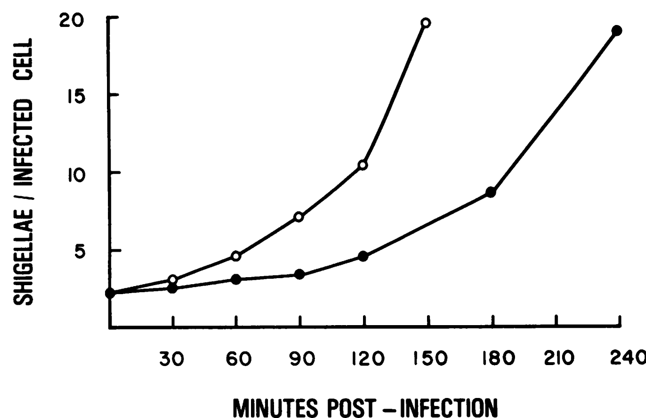

FIG. 2. Intracellular growth of *S. flexneri* 5 at 30 and 37°C. Replicate cultures of HeLa cells previously infected with *S. flexneri* at 37°C were incubated at either 30°C (●) or 37°C (○) in the presence of gentamicin. At various times postinfection, Giemsa-stained preparations of the cultures were examined by light microscopy. The number of shigellae per infected cell was determined by counting a minimum of 500 cells. The percentage of cells infected (25%) did not change over the time course monitored.

did not form plaques. In addition, an *E. coli-Shigella* hybrid (7262-1-10) (13) containing only the 140-megadalton plasmid from *S. flexneri* formed plaques. This indicates that determinants necessary for initial penetration of a host cell, escape from the phagosome, and penetration of subsequent host cells are coded for by this plasmid. It was not clear why the hybrid strains produced smaller plaques than did wild-type shigellae. Possibly some undetermined genetic material is necessary for optimal intracellular replication, or alternatively, perhaps the hybrid strains are more sensitive to gentamicin.

Plaque formation was a result of intracellular multiplication, as gentamicin, an aminoglycoside antibiotic which does not readily kill intracellular bacteria (7, 10), was present in the overlay, thereby killing extracellular shigellae. Similar experiments with *Rickettsia conorii*, an obligate intracellular bacterium, demonstrated that gentamicin did not reduce the plaque titer, although a slight reduction in plaque size occurred (C. Eisemann and E. Oaks, unpublished data). Furthermore, the addition of chloramphenicol or nalidixic acid to the overlay completely inhibited plaque formation by shigellae sensitive to these antibiotics, as these antibiotics are capable of entering the host cell. The lack of plaque inhibition of gentamicin provides evidence that cell-to-cell spread by shigellae occurs before cell lysis. This type of intercellular movement is supported by the ability of shigellae to form plaques in L-cell monolayers that did not contain an agarose overlay but did have gentamicin in the culture medium (E. Oaks, unpublished data).

Temperature had a marked effect on plaque formation by *S. flexneri*. The optimal temperature for the attachment and internalization of shigellae was 37°C. Lower temperatures during the adsorption phase resulted in significant decreases in the number of plaques formed, presumably because the shigellae were unable to penetrate the host cell membrane efficiently at the lower temperatures.

The temperature of growth also had a significant effect on the ability of shigellae to form plaques. *S. flexneri* previously grown at 30°C had a 90% reduction in plaque titer compared with organisms grown at 37°C. This supports the previous findings of Maurelli et al. (11), who showed that the capacity of *S. flexneri* 2 to penetrate Henle cells was dependent on the growth temperature of the shigellae. Furthermore, shigella-infected monolayers initially incubated at 37°C and subsequently shifted to 30°C exhibited a cessation in plaque growth. Plaque dishes initially incubated at 30°C did not form plaques until shifted to 37°C, at which point the plaque size increased at a normal rate although the titer was lower than that of dishes maintained at 37°C. Apparently, temperature-sensitive factors such as the plasmid-coded polypeptides recently shown to be under temperature regulation (T. L. Hale, P. J. Sansonetti, and S. B. Formal, Proceedings of the 19th United States-Japan Cholera Conference, 1983, in press) are involved in the initial penetration of a host cell as well as in subsequent intercellular spreading. At 30°C, the shigellae replicate intracellularly but probably do not enter neighboring cells and are therefore killed by gentamicin upon lysis of the initially infected cell. A few plaques have been observed upon prolonged incubation at 30°C which presumably were the result of mutants no longer temperature sensitive. These strains are currently under investigation.

The plaque assay provides an in vitro investigative procedure for the evaluation of specific virulence factors and their role in pathogenesis. One factor potentially involved in plaque formation is the cytotoxin of *Shigella* species (3, 4) which is capable of inhibiting host cell protein synthesis with subsequent cell death. The high-molecular-weight virulence plasmid which codes for several outer membrane proteins is responsible for the invasion of host cells (13) and also plaque formation. It remains to be determined which of these plasmid proteins are required for the attachment to and eventual destruction of the host cell membrane. Together, the plaque assay and the light microscopy invasion assay make it possible to dissect the phagocytic event from the actual penetration of the host cell membrane by shigellae; i.e., it is conceivable to obtain mutants which appear invasive by light microscopy but do not form plaques. This type of mutant would most likely contain mechanisms for the attachment and stimulation of phagocytosis by the host cell but would lack the equipment required to lyse the phagosomal membrane. The plaque assay also provides a procedure to test the effectiveness of various antibiotics on intracellular shigellae. Currently, the plaque assay is being evaluated as a replacement for the Sereny test as a procedure to isolate virulent shigellae as well as genetically constructed strains carrying the virulence plasmid.

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