Comparative Study of Attachment to and Invasion of Epithelial Cell Lines by \textit{Shigella dysenteriae}

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Henle 407 and HeLa cells were compared as hosts for \textit{Shigella dysenteriae} at a low multiplicity of infection. Efficiency of attachment and invasion without centrifugation, as well as selectivity for pathogenic over nonpathogenic \textit{S. dysenteriae} without Congo red, were much greater for Henle 407 cells than for HeLa cells.

The ability of enteroinvasive bacteria such as \textit{Shigella} spp. to invade cell culture monolayers has been used as a measure of their pathogenicity. Indeed, \textit{Shigella flexneri} and \textit{Shigella sonnei} strains which lose the ability to penetrate HeLa cells are uniformly avirulent in animal hosts (7, 14-16). The invasive phenotype has been mapped to a 220-kilobase extrachromosomal element. Spontaneous deletions in the plasmid lead to noninvasive variants (16). The weak binding between \textit{Shigella} spp. and HeLa cells causes inefficient adherence, and therefore invasion assays have not been highly reproducible. Daskaleros and Payne (3) have improved the efficiency of this interaction for \textit{S. flexneri} by pretreating HeLa cells or bacterial culture with Congo red dye or hemin.

Problems with the invasion assay also arise in assessing the specific location of the bacteria after reaction with the HeLa cells, i.e., whether they are intracellular, residing between the host cells, or attached to the cell monolayers. In order to differentiate adherent bacteria from intracellular organisms, investigators have used a variety of physical, microbiological, and chemical techniques (1, 9, 10, 17). The majority of investigators have used antibiotics to remove or kill adherent noninternalized bacteria (1, 5, 6, 9, 11, 17).

Most studies of invasion mechanisms have used \textit{S. flexneri}, whereas studies with \textit{Shigella dysenteriae} isolates are sparse (2, 4, 5, 10). However, in recent years \textit{S. dysenteriae} has been the causative organism of most of the outbreaks of shigellosis in developing countries, with a high percentage of child mortality (7, 12, 18). We have, therefore, established a sensitive in vitro model for studying invasion by \textit{S. dysenteriae}. Monolayers of the cell line Henle 407 have been found to be susceptible hosts for \textit{S. dysenteriae} infection in the absence of either centrifugation or addition of Congo red and thus provide a useful model for studying the interaction of \textit{S. dysenteriae} with normal host cells.

Fifty strains of \textit{S. dysenteriae} were collected from India, Bangladesh, and other sources. Analysis demonstrated that their large invasion plasmids all had similar sizes (220 kilobases). Invasion plasmids from 10 of these 50 isolates were digested with \textit{EcoRI}. Patterns obtained by agarose gel electrophoresis were identical (12). Accordingly, one isolate, CG097, supplied by J. M. Buyssse from the stocks of D. J. Kopecko, was chosen as the representative virulent organism for our studies. Virulent CG097 (arg trp met Sm') and an avirulent derivative, CG097-2 (arg trp met Sm'), were grown on L agar plus streptomycin (100 \mu g/ml), as these strains are resistant to streptomycin. CG097-2 has lost a 37-kilobase portion of the invasion plasmid and lacks all known \textit{ipa} genes (unpublished data). After every five or six passages on artificial media, bacterial virulence was monitored by the Sereny test as well as by size determination of invasion plasmids (12). Strains which produced keratoconjunctivitis within 48 h were streaked on Congo red-LSB agar (3), to which galactose was added to a final concentration of 0.2%, or L agar plus streptomycin (100 \mu g/ml). Colonies were tested on minimal medium plates, with or without supplements, to confirm the genetic purity of the strains isolated from single colonies.

Cultures were stored at room temperature, rather than at 4°C, for no longer than 1 week. These conditions favor maintenance of intact invasion plasmids. To confirm the presence of intact invasion plasmids, plasmid isolation was performed on a sample of each experimental culture and plasmid size was compared with a known intact standard by agarose gel electrophoresis (12).

Henle 407 cells (embryonic, intestinal epithelial, showing Henle markers, human, Shiga toxin resistant; ATCC CCL 6), Hct8 cells (adenocarcinoma, ileocecal, showing no HeLa markers, human, Shiga toxin resistant; ATCC CCL 244), and a toxin-resistant HeLa cell derivative of ATCC CCL 2 were used as host cells. These lines were maintained in RPMI 1640 containing 10% fetal calf serum, gentamicin (50 \mu g/ml), and 0.01 M HEPES (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) (complete medium), in plastic tissue culture flasks (25 cm²; Corning Glass Works) in 5% CO₂. For passage, the cells were washed and dissociated with 0.25% trypsin in Hanks balanced salt solution. After centrifugation, the pellet was suspended in complete medium and seeded into 24-well plates at 5 \times 10⁴ cells per well. A semiconfluent monolayer of cells was obtained in 24 h. The wells were washed three times with RPMI 1640 to remove antibiotic and fetal calf serum.

Samples of overnight bacterial cultures, grown at 37°C in antibiotic-free L broth, were diluted in RPMI 1640 and added to the wells of tissue culture monolayers at an approximate multiplicity of infection of 5. This multiplicity was chosen, rather than the multiplicities of up to 2,000 used by some workers (5, 14), to minimize nonspecific binding and to increase discrimination between behaviors of different cell lines as bacterial host. In some experiments, the organisms were pretreated with Congo red dye (100 \mu g/ml) at 37°C for 40 min (3) prior to dilution in RPMI 1640. After addition of bacteria, the 24-well plates were either directly incubated at 37°C for 1 h in 5% CO₂ or centrifuged at 2,700 \times g for 5 min and then incubated as above. The wells were then washed four times with phosphate-buffered saline in order to remove...
nonadherent extracellular bacteria. Complete RPMI 1640 containing 50 \( \mu \)g of gentamicin per ml was added, and the plates were incubated for 2 h at 37°C in 5% CO\(_2\). Under these conditions, free CG097 and CG097-2 showed 100% killing in 60 min (data not shown). Monolayer-bacterium complexes were washed twice with antibiotic-free RPMI 1640 and treated with 0.25% trypsin (1) at 37°C for 5 min to dissociate the adherent bacteria, which were then separated from the cells by centrifugation at 1,000 \( \times \) g for 3 min. Supernatants containing these bacteria were plated for viable counts. Intracellular viable organisms were recovered with 0.5% sodium deoxycholate treatment (9) after adherent bacteria were removed. The incubation period with sodium deoxycholate was always less than 30 min, since 0.5% sodium deoxycholate is bactericidal after 30 min of incubation.

In preliminary studies we observed that many \( S. \) flexneri M90T cells invaded HeLa cells, but, with the same ratio of bacteria to HeLa cells, few pathogenic \( S. \) dysenteriae cells invaded the HeLa cells. We then compared Henle 407 and Hct8 cell monolayers with HeLa cells as hosts for attachment of \( S. \) dysenteriae CG097 and its avirulent derivative CG097-2. To test for the effect of centrifugation on attachment, bacteria were added to monolayers and incubated with or without centrifugation. Table 1 presents the data obtained without centrifugation. Many CG097 cells adhered to the Henle 407 cells, while few CG097 cells adhered to the HeLa cells. Stained cover slip preparations confirmed the differences in binding in a qualitative manner (data not shown). Hct8, a human ileocecal cell line, showed adherence which was much higher than that of HeLa but comparable to that of Henle. Furthermore, the selectivity of Henle 407 and Hct8 for CG097 over CG097-2 was much greater than the selectivity shown by HeLa. Centrifugation greatly improved the binding of CG097 to HeLa cells but did not significantly change the binding of the organisms to Henle 407 cells (data not shown).

Since Congo red binding is associated with increased infectivity of \( S. \) flexneri in the HeLa cell model (3), we assessed the ability of \( S. \) dysenteriae, which had prebound Congo red, to invade Henle 407 cells. Invasive \( S. \) dysenteriae CG097 showed neither increased adherence to nor increased penetration of Henle 407 cells in the presence of Congo red (Table 2). Under identical conditions, \( S. \) dysenteriae showed significant increases in both adherence to and penetration of HeLa cells, regardless of the pathogenicity of the organisms. In fact, the avirulent isolate, \( S. \) dysenteriae CG097-2, pretreated with Congo red, showed a 28-fold increase in adherence to and a 9-fold increase in penetration of HeLa cells. The reduction in discrimination between virulent and avirulent organisms, at least as regards invasiveness of HeLa cells by \( S. \) dysenteriae, suggests that the use of Congo red is inappropriate for such studies of \( S. \) dysenteriae.

These data are not necessarily in conflict with the recent suggestion of Sankaran et al. (13) that three membrane proteins, whose concentrations are significantly increased when \( S. \) dysenteriae or \( S. \) flexneri is grown in the presence of Congo red, are virulence proteins. Aside from the fact that their incubation period with Congo red was much longer than that used in our protocol, they did not compare counts of adherent or intracellular organisms with or without Congo red treatment. Such data would be required, particularly at low multiplicities of infection, to establish a link between regulation of one or more of the three membrane proteins and virulence.

Although invasiveness of \( S. \) flexneri (8, 16, 17) and \( S. \) dysenteriae (2, 4, 10) has generally been examined with HeLa cells as an in vitro model, both Henle 407 and Hct8 cells proved more useful than HeLa cells as hosts for \( S. \) dysenteriae on several counts. Efficiency of attachment and of invasion, as well as selectivity for pathogenic over nonpathogenic \( S. \) dysenteriae, for Henle 407 and Hct8 cells was significantly greater than that found for HeLa cells. This increased efficiency, even in the absence of centrifugation, may be due to the presence of high-affinity membrane receptors on these cell lines. Since Henle 407 proved to be a somewhat better host cell line than Hct8, we have chosen to use Henle 407 cells as our in vitro model for study of infection by \( S. \) dysenteriae.

In the course of these studies, we have observed that the large invasion plasmid of \( S. \) dysenteriae CG097 is maintained more stably than that of \( S. \) flexneri M90T (unpublished data). This finding suggests a possible reason for the greater prevalence of \( S. \) dysenteriae in epidemics. However, the fact that even the CG097 invasion plasmid suffers spontaneous deletion of invasion genes makes it imperative to verify the size of the plasmid before each experiment, as was done in

### Table 1. Adherence of \( S. \) dysenteriae to tissue culture lines in the absence of centrifugation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Bacterial inoculum per well</th>
<th>Count of adherent bacteria per well&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>( 5 \times 10^6 )</td>
<td>( 3.5 \times 10^2 )</td>
</tr>
<tr>
<td>Henle 407</td>
<td>( 5 \times 10^6 )</td>
<td>( 3.6 \times 10^3 )</td>
</tr>
<tr>
<td>Hct8</td>
<td>( 5 \times 10^6 )</td>
<td>( 1.3 \times 10^3 )</td>
</tr>
</tbody>
</table>

<sup>a</sup> Count for each line, \( 10^6 \) cells per well.

### Table 2. Effect of Congo red on adherence and invasion of \( S. \) dysenteriae with Henle 407 or HeLa cells as hosts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromogen</th>
<th>Henle 407</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Adherent</td>
<td>Intracellular</td>
</tr>
<tr>
<td>CG097</td>
<td>None</td>
<td>( 3.8 \times 10^4 )</td>
<td>( 4.8 \times 10^4 )</td>
</tr>
<tr>
<td>CG097</td>
<td>Congo red</td>
<td>( 1.3 \times 10^4 )</td>
<td>( 8.9 \times 10^4 )</td>
</tr>
<tr>
<td>CG097-2</td>
<td>None</td>
<td>55</td>
<td>( 1.1 \times 10^3 )</td>
</tr>
<tr>
<td>CG097-2</td>
<td>Congo red</td>
<td>No growth</td>
<td>( 3.3 \times 10^4 )</td>
</tr>
</tbody>
</table>

<sup>a</sup> Time of incubation at 37°C was 1 h without gentamicin and, after washing, an additional incubation for 2 h with gentamicin (50 \( \mu \)g/ml). Adherent bacteria released by trypsinization and internalized (intracellular) bacteria released by sodium deoxycholate were plated for viable colony counts. The data presented are from one of several experiments which generated similar results.
this work. We speculate that the high multiplicities of infection used by many who work with Shigella spp. may, in fact, represent empirical adjustments to compensate for loss of invasion genes by a variable fraction of the bacteria.

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LITERATURE CITED