Enhancement of Invasiveness of *Yersinia enterocolitica* and *Escherichia coli* in HEP-2 Cells by Centrifugation

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Centrifugation enhanced the infectivity of invasive *Escherichia coli* and *Yersinia enterocolitica* for HEP-2 cells. Noninvasive bacteria were not endocytosed after centrifugation. The centrifugation procedure may increase the sensitivity of testing for bacterial invasiveness in cell culture without causing false-positive results.

Invasiveness into epithelial cells is an important pathogenic mechanism of enteric bacteria, including strains of *Shigella*, *Salmonella*, *Escherichia coli*, and *Yersinia* (4, 5, 9, 11). Clinically, the invasive bacteria are capable of producing dysentery-like diseases or exudative diarrhea (1, 7, 13). Demonstration of epithelial invasiveness of *Enterobacteriaceae* can be done by the Serény test (18) or by the use of tissue culture systems, particularly human epithelial cell lines HeLa and HEP-2 (17).

For the bacterial invasiveness test in tissue culture cells, it is vital that test conditions are standardized and that the interpretation of intracellular invasiveness is correct. Mehlman et al. recently described a standard methodology for testing the invasiveness of *E. coli* in HeLa cells and suggested, among other things, that the criterion for a positive invasiveness test in HeLa cells is that 1% of the host cells possess at least five intracellular bacteria (14). The infectivity rate of *E. coli* according to the same authors varies from 0 to 30%. Conceivably then, invasiveness might be missed at a low infectivity rate.

Human pathogenic strains of *Yersinia enterocolitica* are generally invasive in human epithelial cell cultures in vitro (8, 10, 13, 15, 19) or may also show adherence to and a detachment effect on the cells (16, 20). In contrast, human apathogenic biotype 1 strains of *Y. enterocolitica* show no demonstrable interaction with the tissue culture cells (20).

When studying the effect of *Y. enterocolitica* on HEP-2 cells, we observed that invasiveness as well as adherence and detachment effect were greatly enhanced by low-speed centrifugation, but the latter process did not permit the noninvasive strains of *Y. enterocolitica* to invade the cells. The enhancement of sensitivity of the invasiveness test was also seen with invasive strains of *E. coli* and may be applicable to searches for such strains in clinical studies.

The strains of *Y. enterocolitica* used in this study were clinical isolates from the Department of Microbiology, Tampere Central Hospital, and other laboratories in Finland. The strains had been serotyped, biotyped, and tested for the presence of plasmids as described previously (20).

Invasive strains of *E. coli* M4608 and M4163 were gifts from Samuel P. Formal, Walter Reed Army Institute of Research, Washington, D.C.

The standard method which formed the background for the experimental procedures was based on that of Rudoy and Nelson (17) with some modifications. The HEP-2 cells were cultured on glass cover slips in flat-bottom plastic vials (diameter, 13 mm; diameter Sterilin Ltd., Teddington, Middlesex, England). Approximately 50,000 to 60,000 cells were seeded per vial and grown 24 h in Eagle minimal essential medium (Flow Laboratories, Inc., Irvine, Scotland) with 10% fetal calf serum without antibiotics. The cells then appeared in “leopard spot” fashion as a less than confluent monolayer: the total number of cells per cover slip was 150,000 to 200,000. The bacterial suspension at an adjusted concentration was prepared in minimal essential medium, applied onto the cells, and incubated for 3 h at 37°C in an atmosphere containing 5% CO₂. The cells were then washed three times with phosphate-buffered saline and incubated for an additional 2 h in minimal essential medium plus 2% fetal calf serum containing 50 μg of gentamicin per ml. The cells were washed again after 7 h, fixed with methanol, and stained with Giemsa.

In the experiments reported, the procedure was shortened to 6 h and included only one set of three washings at 3 h. The replacement medium contained, in addition to gentamicin, 300 μg of lysozyme per ml.

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The new procedure with centrifugation used the same vials with cover slips and the same quantity of cells. After addition of the bacterial suspension, the vials were centrifuged at 1,000 rpm (212 × g) for 5 or 10 min. After centrifugation, the vials were incubated at 37°C in 5% CO₂ for 1 to 3 h, depending on the experiment. Thereafter, the cells were washed three times with phosphate-buffered saline, and gentamicin- and lysozyme-containing medium was added as in the standard procedure. The cells were fixed and stained 1 to 3 h later.

Judging from the criterion of Mehlman et al. (14), the infectivity rate of Y. enterocolitica usually was between 1 and 5% of the cells. The infectivity rates were obtained by counting 200 random cells per cover slip. After serial cultures at 37°C in the absence of Ca²⁺, infectivity was increased to 30% (20). Centrifugation further enhanced invasiveness to include more than 90% of the cells (Table 1). In a series of 15 strains of Y. enterocolitica serotypes 0:3 and 0:9, the infectivity rate after centrifugation was 91 ± 9%.

Fresh human isolates of Y. enterocolitica serotypes 0:3 and 0:9, which harbored 44- to 47-megadalton plasmids, primarily adhered to and caused a toxic effect on the detachment of the HEp-2 cell monolayer (16, 20), in addition to showing invasiveness. The adherence and the toxic reaction were also enhanced after centrifugation. In striking contrast, the centrifugation procedure did not have any effect on the interaction of the human apathogenic Y. enterocolitica and HEp-2 cells. The eight tested strains were of serotypes 0:6, 0:7/13, and 0:10 belonging to biotype 1. After the invasiveness test with centrifugation, no bacteria were found invasive or adherent to the cells.

Two invasive strains of E. coli were available for testing. In the ordinary test the number of cells invaded by bacteria was 0.5 to 5% at different attempts. The percentage of infected cells increased considerably after centrifugation (Table 1), at best up to 42%. Random stool isolates of E. coli (five were tested) did not show any demonstrable interaction with HEp-2 cells with or without centrifugation.

The mechanism of invasion and intracellular behavior of Y. enterocolitica and E. coli were different. With Y. enterocolitica, a nearly 100% infectivity was obtained after centrifugation, but the bacteria did not multiply appreciably in the cells. Therefore, the test could be terminated at 2 to 2.5 h and the results were similar to those at 6 or 7 h. Lack of intracellular multiplication by Y. enterocolitica was also reported by Devenish and Schiemann (3).

In contrast, the invasive E. coli clearly multiplied in the cells, and a significant difference from an uncentrifuged control test was evident only after several hours of incubation (Table 2). Centrifugation has been used to increase contact between Chlamydia and tissue culture cells to enhance the sensitivity of the isolation procedure (2). Therefore, it appears logical to use a similar method for bacterial infectivity studies. The potential problem is that an artificial pressure might also cause noninvasive bacteria to attach to the cells and to be endocytosed. Our experience with Y. enterocolitica suggested, however, that only true adherence and invasiveness were enhanced by centrifugation, whereas the biotype 1 strains which were indifferent to HEp-2 cells in the regular invasiveness test remained so even after centrifugation. This is also supported by the recent observation of Hale and Formal (6). They found that centrifugation of bacterial suspensions onto HeLa cell monolayers facilitated the uptake or invasive shigellae but did not enhance the uptake of avirulent shigellae. Therefore, bacterial invasiveness tests including centrifugation may not lead to false-positive results.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time (h)</th>
<th>% of cells invadeda</th>
</tr>
</thead>
<tbody>
<tr>
<td>No centrifugation</td>
<td>3c + 3</td>
<td>5</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>1 + 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2 + 2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3 + 2</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3 + 3</td>
<td>42</td>
</tr>
</tbody>
</table>

a Centrifugation was performed at 1,000 rpm for 10 min.

b Means of two parallel cultures.

c The two values indicate the lengths of two incubation times. In between these periods, the medium was changed and the cells were washed.

### TABLE 1. Effect of centrifugationa on the HEp-2 cell infectivity of invasive strains of Y. enterocolitica and E. coli

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>% invasiveness at 6 h</th>
<th>No centrifugation</th>
<th>Centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. enterocolitica 0:3 (cured of plasmid)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6779</td>
<td>34</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>7228</td>
<td>26</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M4608</td>
<td>1</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>M4163</td>
<td>0.5</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

a Centrifugation was performed at 1,000 for 10 min.
The potential applications of the modified invasiveness test remain to be established. As for *Y. enterococolitica*, the centrifugation makes the interaction between bacteria and cells clearer and easier to interpret, and it also shortens the procedure considerably. Devenish and Schiemann recently described another modification of the infectivity test for *Y. enterococolitica* by using cell suspension and a roller bottle apparatus (3). We believe the present method is simpler and more rapid to perform. We also used the centrifugation method to initiate bacterial infection in HEp-2 cells more synchronously for intracellular multiplication studies.

As for *E. coli*, the enhanced sensitivity of the invasiveness test may contribute to the diagnosis of invasive *E. coli* as a cause of exudative diarrhea. In view of the rarity of the latter in Finland (12), we were not able to test this possibility. Centrifugation apparently may also increase bacteria-cell contact of adherent strains of *E. coli*, and it will be important to differentiate between adherence and invasiveness, as is the case in any invasiveness test. Our limited experience with normal stool isolates was again promising, suggesting that such strains remain indifferent in cell culture even after centrifugation and may not interfere with the interpretation of the results in the invasiveness test.

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