Modulation of Bacterial Association to HeLa Cell Cultures by Cell Density and by Chlamydiad Infection

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The effect of cell density on the rate of association of Escherichia coli and Staphylococcus aureus by monolayer cultures of HeLa 229 cells was investigated. Radioactively labeled bacteria were incubated with sparsely and densely plated cells. The rate of bacterial uptake was 10- to 20-fold higher in sparse cultures. Kinetic analysis of data with different multiplicities of input bacteria showed that the $K_m$ of the reaction was unaltered, whereas the $V_{max}$ was inversely related to cell density. Pretreatment of HeLa cultures with dibutyryl adenosine 3',5'-phosphate had little effect on the rate of bacterial association. The simultaneous presence of an obligately parasitic bacterium, Chlamydia trachomatis LGV434, enhanced the $V_{max}$ of association of E. coli and S. aureus. This effect was more pronounced in dense HeLa cell cultures. Heat-inactivated chlamydiae were unable to modify the association. Enhanced association persisted for at least 3 h after infection with chlamydiae.

Members of the genus Chlamydia are obligately parasitic bacteria exhibiting a complex life cycle which is consummated within phagocytic vesicles of the infected host cell. Unlike most pathogenic bacteria which replicate extracellularly, and therefore possess artifacts enabling them to avoid phagocytosis and eventual destruction, the chlamydiae exhibit “parasite-specific” phagocytosis to ensure successful growth. Phagocytosis of chlamydiae, even by nonprofessional phagocytes such as HeLa or L cells, is a highly efficient phenomenon. At moderate nontoxic multiplicities, all of the infectious chlamydiae are phagocytized (5, 6). In view of the presence of chlamydiae in 20% or more of patients with gonorrhea (12), we became intrigued by the possibility that the simultaneous presence of infectious chlamydiae could modulate the uptake of other bacteria by HeLa cells. During the course of this investigation, we also found that the rate of association of Escherichia coli and Staphylococcus aureus was influenced by the cell-to-cell proximity (density) of HeLa 229 cell cultures.

MATERIALS AND METHODS

Cells. HeLa 229 cells were cultured in minimal essential medium supplemented with 5% calf serum which had been heated at 56°C for 30 min.

Bacteria. E. coli B3, a thymine auxotroph, was obtained from N. E. Melechen, and S. aureus 209P was obtained from the Department of Microbiology collection. Both bacteria were grown in glycerol-salts medium (2). E. coli B3 was grown after being supplemented with thymidine (5 µg/ml). The nucleic acids of the bacteria were labeled with [methyl-3H]thymidine (10 µCi/ml), [5,6-3H]uracil, or [3H]cytidine (20 µCi/ml). The bacteria in the exponential growth phase were harvested by centrifugation at 16,000 × g for 5 min in a Sorvall HB4 rotor, washed at least five times with phosphate-buffered saline until no radioactivity was detectable in the wash, and stored at 4°C in Earle balanced salt solution containing 2 mM thymidine, 2 mM uracil, or 2 mM cytidine until used. The specific activity, i.e., counts per minute per viable bacterium, ranged from 0.005 to 0.3.

Chlamydia trachomatis. LGV434/434/Bu (LGV434) strain was grown in HeLa 229 cells as described previously (1). Infected cells, suspended in Earle balanced salt solution with 5% heated fetal calf serum, were stored at −80°C. A rapidly thawed suspension was sheared through a 26-gauge needle to prepare the inoculum for infection.

Measurement of association of bacteria by monolayer cultures of uninfected and chlamydia-infected HeLa cells. In this paper, association of bacteria in which nucleic acids had been labeled with [3H]thymidine, [3H]cytidine, or [3H]uracil is defined as the radioactivity associated with HeLa cells that cannot be removed by repeated washing of the monolayer cultures with phosphate-buffered saline. No attempt was made to distinguish between the attached and the ingested labeled bacteria.

We found that labeled E. coli avidly attached to several tissue culture polystyrene dishes, including multiwell dishes from Lux. However, this did not occur with the 12- and 24-well dishes from Linbro or with the 4-well dishes from Nunc. Monolayers of HeLa cells were detached with trypsin, the cell number was adjusted with antibiotic-free growth medium, and appropriate samples were plated in Linbro or Nunc multi-
well dishes or 3.5-cm-diameter tissue culture dishes. One pair of wells received no cells and served as the blank for background subtraction. After incubation for 1 or 2 days, the monolayers were used for bacterial association experiments. The medium was aspirated, and the monolayer was gently washed twice with Earle balanced salt solution. Labeled E. coli or S. aureus, with or without chlamydia, was added so that the final volume per well was 200 or 500 μl per 3.5-cm-diameter dish. Nonradioactive 2 mM thymidine, 2 mM cytidine, or 2 mM uracil was included to preclude incorporation of any label from the radioactively labeled bacteria during incubation. The multiplicity of chlamydiae was 10 inclusion-forming units per HeLa cell. The multiwell trays were incubated at 37°C while being continuously rocked in a humidified CO2 incubator. The incubation was terminated after 30 min by adding ice-cold phosphate-buffered saline to the wells and then washing each well 10 times with cold phosphate-buffered saline. The cells were scraped with a rubber policeman in 250 μl of phosphate-buffered saline containing 1 mM ethylenediaminetetraacetate. A 100-μl sample was used for the cell number determination, which was measured with a Celsoscope particle counter with a 95-μm orifice. A second 100-μl sample was transferred to a 0.5-dram shell vial containing 10 μl of 0.1 N NaOH to lyse the cells and bacteria. Radioactivity was counted using 1 ml of a Triton X-100-toluene-based scintillant, and the samples were counted with an efficiency of 30% for 3H. The rate of uptake of labeled E. coli was calculated and expressed as counts per minute and then normalized to a constant number of HeLa cells.

RESULTS

Time-dependent association of bacteria with monolayer cultures of HeLa cells. HeLa cells are considered to be nonprofessional phagocytes to distinguish them from macrophages, monocytes, and polymorphonuclear leukocytes which exhibit phagocytic activity several orders of magnitude higher. Between 1 and 2% of the bacterial inoculum became firmly associated with monolayer cultures of HeLa cells in a time-dependent manner (Fig. 1). Therefore, the substrate did not seem to be the rate-limiting step in the association of bacteria with the HeLa cells. The influence of density of HeLa cell cultures on the rate of association of radioactively labeled bacteria is apparent in the data. At any time during the incubation, sparse cell cultures (7 × 10^2 cells per cm^2) showed a 50- to 100-fold higher rate of association than dense cultures (7 × 10^4 cells per cm^2) at two different inputs of [3H]thymidine-labeled E. coli. The rates of bacterial association, using sparse or confluent cultures, were linear for at least 45 min (Fig. 1).

Determination of the apparent kinetic parameters of bacterial association at different HeLa cell culture densities. To examine the possibility that the apparent 50- to 100-fold higher rates of bacterial association to sparse relative to dense cell cultures were merely due to a higher bacterium-to-cell ratio, the association rates of the two types of cell cultures as a function of bacterial input were determined. The dramatic effect of cell density on the rate of association of [3H]thymidine-labeled E. coli is shown in Fig. 2. Even though less than 1% of the bacterial inoculum became cell associated, indicating the availability of excess bacteria for the reaction at the three different inputs and several densities of HeLa cells, sparse cell cultures consistently exhibited 8- to 10-fold higher rates of association, when specific rates normalized to the cell number were calculated. A 10-fold increase in the bacterial inoculum produced less than a 2-fold increase in the rate of association of labeled E. coli with sparse (0.034 × 10^6 cells per well of 2-cm^2 area) cell cultures and a 2- to 3-fold increase in the rate with a 10-fold increase in bacterial inoculum was seen in the dense (2.2 × 10^6 cells per well) cell cultures. To eliminate the possibility that the HeLa cell density-dependent modulation of bacterial association was

![Fig. 1. Kinetics of association of [3H]thymidine-labeled E. coli with sparse (7 × 10^2 cells per cm^2) and dense (7 × 10^4 cells per cm^2) cultures of HeLa 229 cells. One day after being plated in 3.5-cm-diameter dishes, monolayer cultures were infected with 2 × 10^6 (Ο, □) and 1 × 10^8 (Ο, □) cpm of labeled bacteria. Values of uptake are based on the mean of duplicate 35-mm-diameter dishes. To accommodate the data (the association kinetics of the sparse and dense HeLa cell cultures presented with two different bacterial inputs), the rates have been normalized as counts per minute per 10^4 cells for the sparse cell cultures and counts per minute per 10^8 cells for the dense cell cultures.](http://iai.asm.org/)
bacterium specific, radioactively labeled *S. aureus* was used. The data are summarized in Fig. 3 as a double-reciprocal plot which shows the potentially maximal rate ($V_{\text{max}}$) and the $K_m$ of the reactions. It is apparent that with *E. coli* (Fig. 2, inset) and *S. aureus* (Fig. 3), the $K_m$ remained unchanged at the different cell densities. The $V_{\text{max}}$ values were inversely related to the density of cells in the monolayer cultures of HeLa cells. For example, in the experiment shown in Fig. 2, the $V_{\text{max}}$ values were 4,255, 2,128, 447, and 266 cpm per $10^5$ HeLa cells at cell densities of $0.017 \times 10^6$, $0.05 \times 10^6$, $0.38 \times 10^6$, and $1.1 \times 10^6$ cells per cm$^2$. The density-dependent modulation of bacterial association was repeatedly observed when several radioactive precursors, e.g., thymidine, cytidine, uracil, amino acids, and $^{38}P$, were used to label bacterial macromolecules.

It should be noted that in the association studies documented here, excess unlabeled precursors (thymidine, cytidine, uracil, and amino acids) were included in the reaction mixtures to effectively eliminate the incorporation of any unstable label from the bacteria into multiple-washing-resistant material. Moreover, the association rates of both bacteria to the monolayer cultures were time, multiplicity, and temperature dependent.

**Lack of effect on bacterial association by pretreatment of HeLa cell cultures with dibutyryl cAMP and isobutylmethylxanthine.** Inhibition of phagocytosis by dibutyryl adenosine 3',5'-phosphate (cAMP) (8, 11) and enhancement (15) have been reported. The growth of HeLa 229 cells is inhibited by exogenous cAMP (19), and high intracellular levels of cAMP in nongrowing cultures of "normal" cells (16) have been observed. Repeated attempts failed to influence significantly the rate of association of *E. coli* or *S. aureus* with sparse or dense cultures of HeLa 229 cells by treatment with 2.5 or 5 mM dibutyryl cAMP, with or without isobutylmethylxanthine, an inhibitor of cAMP phosphodiesterase (Table 1).

**Effect of infection by *C. trachomatis* on the rate of association of *E. coli* or *S. aureus* with HeLa cell cultures.** The rates of associ-
BACTERIAL ASSOCIATION AND HeLa CELL DENSITY

TABLE 1. Lack of effect of treatment of HeLa 229 cell cultures with dibutyryl cAMP and isobutylmethylxanthine on the rate of association of labeled S. aureus

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Culture density</th>
<th>Association of [3H]lactococcal-labeled S. aureus (cpm per 10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Low</td>
<td>1.5 x 10^4</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2.6 x 10^3</td>
</tr>
<tr>
<td>2.5 mM dbcAMP</td>
<td>Low</td>
<td>1.5 x 10^4</td>
</tr>
<tr>
<td>2.5 mM dbcAMP</td>
<td>High</td>
<td>3.5 x 10^3</td>
</tr>
<tr>
<td>+ 1 mM IBMX</td>
<td>Low</td>
<td>1.1 x 10^4</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>2.8 x 10^3</td>
</tr>
</tbody>
</table>

* dbcAMP, Dibutyryl cAMP; IBMX, isobutylmethylxanthine.

Mydia-suspending medium, and heat-inactivated chlamydiae were unable to effect the enhancement (Table 2). Chlamydia-specific enhancement persisted for at least 3 h after infection of HeLa cells (Table 2).

Kinetic analysis of data obtained with [3H]cytidine-labeled S. aureus showed that the presence of infectious chlamydia increased the V_{max} of the rate of association of labeled bacteria from 65 cpm per 10^4 HeLa cells to 130 without a detectable change in the apparent K_{m} of the reaction (Fig. 5).

![Double-reciprocal plot of the rate of association of [3H]cytidine-labeled S. aureus as affected by the infection of confluent monolayer cultures of HeLa cells with C. trachomatis LGV434.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on August 27, 2017 by guest)

![Influence of chlamydial infection on the cell density-dependent association of E. coli.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on August 27, 2017 by guest)
DISCUSSION

Several membrane-related functions are modulated by the proximity of cells growing in vitro. An inverse relationship of cell density with regard to the transport of hexoses, the level of glycolytic enzyme activity, glucose utilization, and lactate production has been described (3, 9). A similar inverse relationship between receptor-mediated uptake of low-density lipoprotein and cell density was recorded with bovine endothelial cells (22) and human skin fibroblasts (14) grown in vitro. Pinocytosis of horseradish peroxidase, on the other hand, was enhanced in dense, relative to sparse, cultures of L (20), HeLa, and monkey kidney (13) cells. The data summarized in Fig. 2 and 3 show that the rate of association of E. coli and S. aureus to HeLa 229 cells was dramatically reduced in dense, relative to sparse, cell cultures. By increasing the bacterial input, the rate of association to dense cultures of HeLa cells could not be enhanced to the rate observed with sparse cultures (Fig. 2 and 3). Thus, simple increases in the bacterium-to-HeLa cell ratio did not result in the observed higher rate of association of labeled bacteria with the sparse cultures of HeLa cells. Lineweaver-Burk plots have been used to analyze phagocytic activity (17, 21). The $V_{\text{max}}$ of a phagocytic reaction is thought to reflect the avidity of cell-particle interaction at the membrane, whereas the $K_m$ is a measure of the phagocytic capacity of the cell (17). The enhanced $V_{\text{max}}$ resulting from opsonization of $[^{3}H]$thymidine-labeled S. aureus was interpreted to reflect increased bacterium-macrophage interaction at the cell surface (17). In the same study, depression of the $K_m$ by several inhibitors led to the postulate that the $K_m$ could not represent affinity of the cell for the bacterium, but instead was an index of the phagocytic capacity of the cell. Our data from many different experiments indicate a cell density-dependent change only in the $V_{\text{max}}$ of association of bacteria with the HeLa cell cultures. Such a change suggests modulation of the avidity (17) of cell-particle interaction by the proximity of the cell and not the capacity of the cell for uptake.

The lack of effect of treatment with dibutyryl cAMP noted in our study (Table 1) suggests that although inhibition of growth of HeLa 229 cells by cAMP may occur (19), the cyclic nucleotide has no immediate influence on the rate of association of two different bacterial species. Lack of influence on the association of labeled infectious elementary bodies of C. trachomatis LGV434 by dibutyryl cAMP has also been observed (S. K. Bose and R. Paul, manuscript in preparation). At present, therefore, the density-dependent modulation of bacterial uptake by HeLa 229 cells does not appear to be under the direct control of cAMP.

With polymorphonuclear leukocytes (7) and mouse peritoneal macrophages (18), it has been shown that phagocytosis of opsonized or heat-inactivated staphylococci results in the enhancement of phagocytosis of bacteria or glutraldehyde-treated erythrocytes. On the other hand, phagocytosis of latex beads or opsonized pneumococci has little effect on the phagocytosis of mouse erythrocytes attached via F(ab')2 immunoglobulin fragments to mouse macrophages (10). Heat-labile surface components of the elementary bodies of Chlamydia psittaci are responsible for the highly efficient parasite-controlled phagocytosis leading to successful infection (4–6). The uptake of heat-inactivated chlamydiae, however, is not enhanced by the presence of infectious elementary bodies (4), a phenomenon similar to the lack of ingestion of a non-phagocytizable organism by leukocytes which have ingested other bacteria (7). HeLa cell cultures with high phagocytic activity, i.e., sparse cell cultures, showed less enhancement of association of E. coli in the presence of chlamydiae than did the dense cell cultures (Fig. 4). The $V_{\text{max}}$, but not the $K_m$, of the rate of association of S. aureus was increased in the presence of the chlamydial inoculum (Fig. 5). Membrane fluidity and the intracellular cyclic nucleotide level are only two of many parameters which play a major role in functions associated with the cell surface. Both of these are affected by cell proximity.

Because little is known about bacterial receptors on the plasma membrane, especially their local distribution and the influence of membrane fluidity on bacterial association, one can only speculate that the sparse cultures possess greater avidity regarding bacterial binding. Clustering of receptors on the plasma membrane may be one of the factors responsible for the enhanced association of bacteria exhibited by sparse cultures of HeLa cells. Infection with C. trachomatis LGV434, an obligate parasite with a highly efficient mode of association to HeLa cells, may cause a more optimal orientation of the receptors, resulting in the observed enhancement of the rate of association of E. coli and S. aureus.

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