Distribution of the Receptor Sites for Sindbis Virus on the Surface of Chicken and BHK Cells

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Received for publication 15 April 1974

Sindbis virus was adsorbed to chicken cells or to BHK cells, and the distribution of virus over the surface of the cell was examined by electron microscopy of surface replicas. The distribution of virus particles on the cell was used to indicate the position of virus receptors at the cell surface. When purified Sindbis virus was adsorbed at 37°C to cells prefixed with glutaraldehyde, the virus particles were evenly distributed over the surface of most cells. There was a large variability from cell to cell, however, in the number of virus particles adsorbed, and regions with different concentrations of virus particles were sometimes observed on the same cell. The concentration of virus receptors observed varied from 20 to 160/μm² of cell surface, and, thus, the total number of virus receptors per chicken cell is on the order of 10⁴. When virus was adsorbed to unfixed cells at 4°C, the virus particles were clustered into aggregates varying from a few particles to large crystalline arrays (the latter seen only in chicken cells). These conditions are apparently conducive to virus aggregation, and this, coupled with free lateral diffusion of the virus-receptor complex in the cell membrane at 4°C, leads to the observed clustering.

The early events in the infection of cells by animal viruses are the adsorption of the virion to a receptor on the cell surface, loss or eclipse of the virus particle infectivity, and penetration of the virus into the cell (1, 13, 15). Adsorption occurs at 0°C, but eclipse does not. At higher temperatures (20°C or above), eclipse and penetration occur. In the case of poliovirus and of several other viruses with a high particle-to-PFU ratio, a large fraction of the eclipsed particles can be washed off the cell; these particles contain all of the macromolecules of the virus, but they will not reabsorb to the cell (5, 8, 15). There seem to be two ways for the virion to penetrate the cell: pinocytosis (4, 14), followed by the breakdown of the virus inside the cell, or fusion of the membrane of the virus with the plasma membrane of the cell and release of the viral nucleic acid (20).

The virus receptors on the cell surface are quite specific. For example, the adsorption of influenza virus at 4°C to erythrocytes is a specific reaction, based on the differences in, hemagglutination for different species of erythrocytes (3, 11). The adsorption of the picornaviruses also involves binding to a specific receptor, based on two lines of evidence. First, poliovirus infects only a few different cell types in vivo. However, some cells not susceptible to poliovirus in vivo become so after culturing in vitro (12); this susceptibility is accompanied by the appearance of virus receptors. Second, receptors specific for several picornaviruses have been distinguished from each other on the basis of sensitivity to enzyme inactivation and time required for the regeneration of receptor activity (17, 22, 28). In the case of adenovirus, the specificity of the receptor has also been established (23). Although the specificity of virus-receptor interactions is quite certain, little is known about the structure of virus receptors. For the myxoviruses and paramyxoviruses, sialic acid appears to be part of the receptor (10); the receptor of influenza virus in erythrocyte membranes seems to be a glycoprotein (16, 19).

Studies thus far on the adsorption of viruses to cells have not examined the distribution of virus receptors on the cell surface. We have successfully used the surface replica technique to study the topography of Sindbis virus-infected cells (2), and we have now used this technique to study the distribution of Sindbis virus receptors on chicken embryo fibroblasts and on BHK-21 cells.

(This work was taken in part from a Ph.D. thesis submitted by C.R.B. to the California Institute of Technology, Pasadena, 1974.)
MATERIALS AND METHODS

Cells. The culturing of chicken embryo fibroblasts and their growth on 12-mm cover glasses have been described previously (2). BHK-21 cells were cultured in Eagle medium containing 10% fetal calf serum.

Adsorption of virus to cells. Sindbis virus (HR strain) was purified by polyethylene glycol precipitation followed by velocity and isopycnic sucrose gradients, as previously described (24, 27), and dialyzed overnight at 4 C against phosphate-buffered saline (PBS), pH 7.4 (7). The virus solution before dialysis had a protein concentration of 1 mg/ml, as determined by the Lowry assay (18). After dialysis, fetal calf serum was added to a concentration of 1%, and the virus was further diluted with PBS containing 1% fetal calf serum to protein concentrations of 25, 50, 125, and 250 μg/ml.

For adsorption at 37 C, all manipulations were done in a 37 C room, and all cells were prefixed before adsorption to prevent movement of the virus receptors. Cells were washed in PBS, fixed in 1% glutaraldehyde in PBS for 10 min at 37 C, and then washed again in PBS. To remove unreacted glutaraldehyde, cells were treated with .15 M glycine in PBS for 10 min at 37 C. After being washed again, cells on cover glasses were treated with 0.1 ml of a virus dilution for 30 min at 37 C and then washed several times in PBS.

For adsorption at 4 C, all manipulations were done in a 4 C room. Before adsorption, cells were allowed to cool to 4 C in PBS with 1% fetal calf serum. Except for the temperature difference, the procedures for fixation and adsorption at 4 C were the same as those at 37 C.

Electron microscopy. Fixation of cells and preparation of surface replicas have been previously described (2). All micrographs are presented in reverse contrast.

RESULTS

Since the adsorption of virus to cell surfaces involves attachment to a specific receptor, we have used the distribution of adsorbed Sindbis virus on the cell surface to determine the distribution of Sindbis virus receptors. Adsorbed virus particles will penetrate the cell at 37 C, so the adsorption of Sindbis virus was studied under conditions which inhibit penetration into the cell, i.e., adsorption at low temperatures or prefixation with glutaraldehyde before adsorption.

In the following sections, density refers to the number of virus receptors or virus particles per square micrometer of the cell surface.

Adsorption of virus at 37 C. For these experiments, cells were prefixed at 37 C before adsorption. A surface replica of two chicken cells, A and B, after adsorption of Sindbis virus at 37 C is shown in Fig. 1. The cells are partially overlapping; the solid and black-and-white arrows indicate the edges of cells A and B, respectively. These cells illustrate two points about the distribution of Sindbis virus receptors. First, receptors were evenly distributed over the entire cell surface; even pseudopodia extending from the cell periphery were uniformly covered with virus particles. Second, the density of receptors on the cell surface varied from cell to cell. In Fig. 1, cell A has a density of 96 virus particles per μm², whereas cell B has a density of 46 virus particles per μm². Virus particle densities on other cells were found to vary from about 20 to 160 per μm². The virus receptors were saturated under the conditions used, since the same results were obtained with all the virus dilutions used. Assuming that chicken embryo fibroblasts in tissue culture have an average surface area of 2 × 10⁴ μm², the number of Sindbis virus receptors on these cells varies from 4 × 10⁴ to 3 × 10⁶. This probably represents only a minimal value, since virus particles may bind to or overlap more than one receptor molecule.

The adsorption experiments with chicken cells have been done on secondary and tertiary cultures of cells, as well as on primary cultures. The results were approximately the same in every case. Thus, the variability in density of virus particles adsorbed is unlikely to be related to variability of cell types in the primary culture, since secondary and tertiary cultures are far more uniform in cell population.

We also adsorbed virus to BHK cells under these same conditions. As with chicken cells, virus was evenly distributed over the cell surface and the density of adsorbed particles was variable from cell to cell. The variation in virus density observed was 25 to 150 particles per μm², with most cells exhibiting a density of 100 to 150 particles per μm². This range in adsorption density of virus particles is very close to that seen with chicken cells.

Although most cells had the same density of virus particles over their entire surface, areas with different particle densities were sometimes observed on the same cell, as shown in Fig. 2. The denser area of this chicken cell has a density of 77 virus particles per μm², whereas the less dense area has a density of 20 virus particles per μm².

Adsorption of virus at 4 C. When virus was adsorbed to cells at 4 C, there was a large variability from cell to cell in the density of virus particles on the cell surface, as had been seen at 37 C. In contrast to the even distribution of virus particles seen on prefixed cells adsorbed at 37 C, however, unfixed cells adsorbed at 4 C showed a clustering of virus particles on the cell surface. A surface replica of two unfixed chicken cells adsorbed at 4 C is shown in Fig. 3; as in Fig. 1, these cells are also overlapping. The solid
FIG. 1. Surface replica of Sindbis virus adsorbed to prefixed chicken cells at 37 C. The solid and black-and-white arrows indicate the edges of cells A and B, respectively. ×14,500. Bar, 1 μm.
FIG. 2. Surface replica of Sindbis virus adsorbed to a prefixed chicken cell at 37 C. ×14,500. Bar, 1 μm.
Fig. 3–5. Surface replicas of Sindbis virus adsorbed to unfixed chicken cells at 4 C. Bars, 1 μm.

Fig. 3, ×17,000. The solid and black-and-white arrows indicate the edges of cells A and B, respectively.

Fig. 4, ×18,000.

Fig. 5, ×39,000.
and black-and-white arrows indicate the edges of cells A and B, respectively. Cell A has a density of 200 virus particles per \( \mu m^2 \) on its surface, whereas cell B has a density of 50 virus particles per \( \mu m^2 \). The virus particles on cell A are aggregated into large clusters in which the virus is tightly packed, whereas the clusters on cell B are much smaller and there are many single particles. At an intermediate density, the clustering was more apparent, as on the chicken cell in Fig. 4, which has a density of 150 virus particles per \( \mu m^2 \) on its surface. On a few chicken cells the clustering at 4 C was so extensive that the virus particles appeared to form a crystalline lattice on the cell surface (Fig. 5). The large cluster seen in Fig. 5 has a density of 450 virus particles per \( \mu m^2 \). At this density, the virus particles have an effective diameter of only 60 nm, assuming hexagonal close packing, which indicates that in such clusters the particles are very tightly packed since the measured diameter of a Sindbis virion is approximately 70 nm.

Virus adsorbed to BHK cells at 4 C also showed considerable clustering, although clustering to the extent shown by the cell in Fig. 5 was not seen in BHK cells.

There seem to be three possible explanations for this clustering of virus particles at 4 C. First, Sindbis virus particles aggregate in solution at 4 C and, therefore, adsorbed as aggregates at this temperature. Second, cooling cells to 4 C causes the virus receptors to aggregate or "freeze out" of the plasma membrane. Third, after virus particles bind to receptors on the cell surface, the virus-receptor complexes migrate laterally along the cell surface to form aggregates. The following experiments were done with chicken cells to test these possibilities (Table 1). One set of cells was prefixed at 37 C, cooled, and adsorbed with Sindbis virus at 4 C. Two other sets of cells were cooled and prefixed at 4 C; one set was then warmed and adsorbed at 37 C, whereas the other was adsorbed at 4 C. Adsorption at 37 C of cells prefixed at 37 C and adsorption of unfixed cells at 4 C were used as controls, since it was known that under these conditions the virus particles had even and clustered distributions, respectively. Adsorption at 37 C to unfixed cells was not attempted because of the complications in interpretation caused by virus penetration.

All conditions of adsorption used produced an even distribution of virus particles, except adsorption at 4 C of unfixed cells (Table 1). If the virus had aggregated in solution at 4 C, then adsorption at 4 C of cells prefixed at either 4 or 37 C would have produced a clustered distribution of particles. If cooling cells to 4 C clustered the virus receptors, then adsorption at either 4 or 37 C of cells cooled and prefixed at 4 C would have also produced a clustered distribution. Therefore, it appears that the virus receptors can diffuse laterally in the plasma membrane even at 4 C.

### DISCUSSION

Fixation of cells with glutaraldehyde inhibits diffusion of proteins within the plasma membrane and prevents the penetration of adsorbed virus into the cell, thus allowing one to examine the true distribution of virus receptors on the cell surface by adsorbing virus onto prefixed cells. Using this procedure, we have found that Sindbis virus receptors are randomly distributed over the surface of chicken embryo fibroblasts and BHK cells. Random distributions have also been reported for other cell surface antigens, such as the concanavalin A-binding sites on normal and transformed cells (21, 26).

The density of Sindbis virus receptors on the cell surface varies from cell to cell, ranging from 20 to 160 virus particles adsorbed per \( \mu m^2 \) on prefixed cells adsorbed at 37 C. Areas of different particle densities were sometimes found on the same cell. Variability in the number of influenza virus particles adsorbed to endodermal cells of the choroidallantoic membrane of chicken embryos has been reported (20), but as thin-sectioning techniques were used in this study, the observed variability may not apply to the whole cell surface. It is not clear why different cells have different numbers of Sindbis virus receptors on their surfaces, but this may be partly due to the asynchrony of the cell population.

The virion-receptor complex could diffuse laterally over the cell surface at 4 C in unfixed cells, leading to clustering of the virus particles on the cell surface. This clustering appears to be due to a tendency for the virus to crystallize or aggregate under these conditions, since the receptors do not clump in the absence of virus. It is known that low temperature inhibits the

### Table 1. Adsorption of Sindbis virus to chicken embryo fibroblasts

<table>
<thead>
<tr>
<th>Preadsorption treatment</th>
<th>Temp of adsorption (C)</th>
<th>Distribution of virus particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefixed at 37 C</td>
<td>4</td>
<td>Even</td>
</tr>
<tr>
<td>Prefixed at 37 C</td>
<td>37</td>
<td>Even</td>
</tr>
<tr>
<td>Prefixed at 4 C</td>
<td>4</td>
<td>Even</td>
</tr>
<tr>
<td>Prefixed at 4 C</td>
<td>37</td>
<td>Even</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>Clustered</td>
</tr>
</tbody>
</table>

Note: This table presents the conditions of adsorption and the resulting distribution of virus particles on chicken embryo fibroblasts, as detailed in the DISCUSSION section.
movement of certain antigens on the cell surface (9), but there are examples of such movement at low temperature. Treating mouse lymphocytes at 0°C with divalent antibodies against mouse immunoglobulin G produces a patchy distribution of immunoglobulin G molecules on the cell surface, although capping (movement of cell surface immunoglobulin G to one pole of the cell) is inhibited at this temperature (6). Also, the intramembranous particles of erythrocyte membranes can be aggregated at low temperatures (25). Thus, when studying the cell surface distribution of any antigen, one cannot assume that low temperatures prevent movement of the particular antigens under study.

ACKNOWLEDGMENTS

We are grateful to Jean-Paul Revel for his advice throughout the conduct of these experiments, and to Ellen Strauss for her aid in preparing the manuscript. Edith Lenches and Sharman Christoph provided excellent technical assistance.

This work was supported by Public Health Service grant GM 06965 from the National Institute of General Medical Sciences and by grant GB 31763X from the National Science Foundation. C. R. B. was supported by Public Health Service Training Grant GM00086 from the National Institute of General Medical Sciences.

LITERATURE CITED