Sequential Steps in Attachment of Human Rhinovirus Type 2 to HeLa Cells

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(Accepted 10 June 1974)

SUMMARY

When human rhinovirus type 2 attaches to HeLa cells, it first forms a reversible complex which can be dissociated by addition of EDTA to the medium. After prolonged incubation at 25 °C, most of the cell-associated virus progresses to an irreversible complex from which it cannot be eluted by EDTA, although some noninfectious material elutes spontaneously. The addition of a sufficient concentration of SDS to block cell-mediated eclipse of the virus does not block the formation of the irreversible complex at 25 °C, while virus adsorbed and incubated at 0 °C remains reversibly bound. The number of receptor sites per cell is not influenced by SDS, but at 0 °C cells appear to have fewer receptor sites than at physiological temperature.

INTRODUCTION

The nature of the reaction leading to the attachment of picornaviruses to cells is poorly understood. Virus-receptor interaction resembles antibody-antigen interaction in its specificity and in the lack of formation of covalent bonds (reviewed recently by Lonberg-Holm & Philipson, 1974). However, the basis for the peculiar temperature dependency of the rate of attachment of some picornaviruses, including rhinoviruses and polioviruses (Lonberg-Holm & Korant, 1972), has not been explained.

It has been established that there are a limited number of cellular receptors for each rhinovirus studied thus far. After about $10^4$ virus particles have attached per cell, covering roughly 1% of the cell surface, the rate of attachment of additional particles is markedly reduced. This has permitted the assignment of individual serotypes of rhinoviruses which share a common receptor to 'receptor families' (Lonberg-Holm & Korant, 1972; K. Lonberg-Holm, unpublished observations). Attachment of purified virus particles of human rhinovirus type 2 (HRV-2) to cells is relatively efficient at physiological temperatures and is blocked by the addition of 5 mM-EDTA to the medium (Lonberg-Holm & Korant, 1972).

Cell-associated rhinovirus particles can be recovered in infectious form by the lysis of the infected cells with detergents. The steps leading to cell-mediated 'eclipse' of HRV-2 particles to produce non-infectious particles have been elucidated (Lonberg-Holm & Korant, 1972; Lonberg-Holm & Noble-Harvey, 1973). In vitro methods have also been developed to produce altered particles which resemble those formed by the cell...
(Korant et al. 1972), and it has been shown that these alterations produce profound antigenic changes (Lonberg-Holm & Yin, 1973) which correlate with loss of the ability of the altered particles to attach to cellular receptors (Noble & Lonberg-Holm, 1973). An important finding used in these studies was the discovery that $10^{-4}$ M-SDS blocks the cell-mediated 'eclipse' of HRV-2 without significantly altering the rate of virus particle attachment (Lonberg-Holm & Noble-Harvey, 1973).

The comprehensive understanding of the early events in infection by HRV-2 now permits a closer examination of the initial attachment to host cells. We report here evidence for a sequential change in the binding of infectious virus to the cell.

**METHODS**

**Virus and cells.** The origin, propagation and purification of [14C]-amino acid-labelled HRV-2 has been described (Korant et al. 1972). Virus was dialysed against medium (see below), after 5% heat-inactivated foetal calf serum was added. The relationship between radioactivity and the number of physical particles was known in each case (Korant et al. 1972). HeLa cells for use in adsorption studies were grown in suspension culture and were handled as already described (Lonberg-Holm & Korant, 1972).

**Medium.** Eagle’s minimal essential medium, modified for spinner culture, and supplemented with 5% heat-inactivated foetal calf serum (56 °C for 1 h) was used in all experiments.

**Adsorption of virus to cells.** Attachment of HRV-2 to HeLa cells in suspension culture at 25 °C and 34.5 °C, and the determination of the percentage of cell-associated radioactivity was carried out as previously described (Noble & Lonberg-Holm, 1973). In this method, the percent cell-associated radioactivity is calculated by dividing the cell-associated counts by the sum of the cell-associated counts, the counts in the supernatant fluid, and the counts in one wash of the cells.

HRV-2 was adsorbed to HeLa cells at 0 °C as follows. The cells were washed prior to use as described before and resuspended in sufficient medium to give $5 \times 10^7$ cells/ml after addition of virus. Cells were normally kept at 0 to 7 °C for 1 h or longer before the addition of virus. After all components had equilibrated to 0 °C in an ice bath, virus was added to give the multiplicities indicated in individual experiments. The suspension was then agitated by magnetic stirring in an ice bath and 0.3 to 0.5 ml samples were removed as indicated. Cell-associated virus was assayed by the same procedures used at higher temperatures.

**Elution of virus.** HeLa cells with attached virus were washed twice with medium at 0 °C by low-speed sedimentation and resuspended in medium at 0 °C, before reincubation at the temperature specified in each experiment. Cell-associated radioactivity was determined after various periods of elution, as described previously (Noble & Lonberg-Holm, 1973). The medium used during elution sometimes contained EDTA or SDS, as indicated for the individual experiments. EDTA was not present during washing when it was present during elution. Cell-associated HRV-2 showed the same behaviour when exposed to medium containing 5 mM- or 10 mM-EDTA (data not shown). At both concentrations, EDTA is present in excess of the amount necessary to chelate the divalent cations present in the medium.

In the graphic representation of elution data, the cell-associated radioactivity immediately after washing and resuspension of the cells in medium was normalized to 100%. The actual values were more than 95% in all cases.

**Infectivity assay.** To titrate the infectivity of cells and supernatant fluid of an 0.5 ml
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Fig. 1. Elution of HRV-2 from HeLa cells in medium containing EDTA. [14C]-amino acid-labelled HRV-2 was adsorbed to HeLa cells in medium at 25 °C at a multiplicity of 10^4 particles/cell for 1 min (a) or for 20 min (b). Approx. 10% of the original radioactivity was cell-associated after 1 min, and about 70% after 20 min. The cells were then washed and resuspended for elution in medium (○—○) or medium containing 10 mM-EDTA (○—○—○). Samples of 0·5 ml were removed at 10 min intervals during elution at 25 °C, and the percentage cell-associated radioactivity in each was determined (Methods).

sample, the cells were sedimented by low-speed sedimentation at 4 °C and the supernatant medium was decanted. The cells were then washed with 4 vol. of medium at 0 °C, and re-suspended in 0·02 M-tris-HCl buffer, pH 8·1, at 0 °C (final vol. of 2·0 ml). Samples of 0·2 ml of the resuspended cells or 0·2 ml of the supernatant fluid were mixed with 0·2 ml of tris buffer, pH 8·1, containing 0·8% SDS. These were held at room temperature for 10 min, then diluted to 4·0 ml with cold medium and stored at —70 °C until used for the plaque assay of virus infectivity (Korant et al. 1972).

RESULTS

Elution of HRV-2 from HeLa cells

It has already been reported that radioactive HRV-2 elutes spontaneously from HeLa cells after prolonged incubation at 34·5 °C (Lonberg-Holm & Korant, 1972; Noble & Lonberg-Holm, 1973). Much of the radioactivity which elutes spontaneously resides in non-infectious particles which sediment more slowly than virus particles and resemble the major product of cell-mediated eclipse (Lonberg-Holm & Korant, 1972). Both attachment and subsequent elution of radioactive HRV-2 have now been examined at a lower temperature, in order to slow the early events of virus-cell interaction.

Fig. 1 shows the pattern of elution of HRV-2 at 25 °C, after attachment at the same temperature, either in normal medium or in medium containing EDTA. Elution of HRV-2 after brief periods of virus-cell interaction differed significantly from elution after longer periods of adsorption. After attachment for 1 min (Fig. 1 a), no detectable cell-associated virus was eluted during 30 min of subsequent incubation in medium alone; while about 25% eluted with EDTA present. Of the virus which had attached during 20 min (Fig. 1 b), about 7% eluted in the absence of EDTA while about 14% eluted in the presence of EDTA. This difference shown in Fig. 1 b is statistically significant because the total radioactivity in each sample was approx. 5400 ct/min net; and the cellular, supernatant, and wash fractions of each sample were counted separately for a total of 20 min.
Table I. EDTA-induced elution of infectious HRV-2 from HeLa cells*

<table>
<thead>
<tr>
<th>Adsorption at 25 °C</th>
<th>Infectivity†</th>
<th>Radioactivity†</th>
<th>Elution at 25 °C</th>
<th>Sample</th>
<th>% original infectivity†</th>
<th>% original radioactivity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>12</td>
<td>9-6</td>
<td>30 min, medium</td>
<td>Cells</td>
<td>5-0</td>
<td>9-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fluid</td>
<td>&lt;0-1</td>
<td>0-1</td>
</tr>
<tr>
<td>20 min</td>
<td>39</td>
<td>68</td>
<td>30 min, medium+ 10 mM-EDTA</td>
<td>Cells</td>
<td>3-3</td>
<td>8-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fluid</td>
<td>2-7</td>
<td>3-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 min, medium+ 10 mM-EDTA</td>
<td>Cells</td>
<td>8-8</td>
<td>63-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fluid</td>
<td>0-2</td>
<td>5-5</td>
</tr>
</tbody>
</table>

* Data are from an experiment similar to that shown in Fig. 1. Infectivity and radioactivity in the cells and in supernatant fluids were assayed as described in Methods.
† Values were determined from samples of the cell-virus mixture at 0 °C, before adsorption at 25 °C. Original infectivity was 4.8 \times 10^6 p.f.u./ml; original radioactivity was 1.6 \times 10^4 ct/min/ml.

Infectious material eluted from cells

Experiments similar to that described above showed that virus material which dissociated from cells after brief periods of adsorption, using 5 mM-EDTA in the medium, could adsorb to cells again, after the medium was supplemented with 10 mM-Mg^{2+} (unpublished data). This observation suggested that infectious virus had eluted, and this was confirmed by plaque assay.

Table 1 compares the radioactivity and infectivity which was cell-associated or in the supernatant medium, after adsorption of [^{14}C]-labelled HRV-2 to HeLa cells at 25 °C for 1 or 20 min, followed by elution for 30 min at 25 °C in medium alone or medium containing 10 mM-EDTA. Virus eluted with EDTA after 1 min of attachment comprised 31% of the original radioactivity added to the cells and 27% of the original infectivity. Hence, 80 to 90% of the eluted virus was infectious. After adsorption for 20 min, little, if any, of the virus material eluted in the absence of EDTA was infectious, while 30 to 40% of that eluted with EDTA was infectious.

From the data of Table 1 it is evident that EDTA has little effect on the loss of cell-associated infectivity during the elution period, except that some infectious virus dissociates from cells, and therefore escapes cell-mediated inactivation.

Attachment and elution of HRV-2 at 0 °C

Although adsorption of HRV-2 to HeLa cells is temperature dependent (Lonberg-Holm & Korant, 1972), attachment does occur at 0 °C. The extent of this depends somewhat upon the length of time cells are held at 0 °C before virus is added (unpublished results). Before starting the experiments described here, cells were cooled during washing and handling for about 1 h, as described in Methods. Adsorption is largely complete by 60 min under the conditions of high cell concentration (5 \times 10^7/ml) and low input multiplicity (2 \times 10^3 particles per cell) employed (Fig. 2a). The amount of cell-associated virus increased only by about 10% during the period from 60 to 90 min after the start of adsorption at 0 °C (see Fig. 4a).

After adsorption to cells at 0 °C, the major portion of the cell-associated virus could be
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![Graph showing adsorption and elution of HRV-2 at 0 °C.](image)

Fig. 2. Adsorption and elution of HRV-2 at 0 °C. (a) shows the net adsorption of [14C]-amino acid labelled HRV-2 to HeLa cells (5 x 10⁷/ml) at 0 °C at a multiplicity of 2 x 10⁶ particles/cell (see Methods). (b) shows the elution at 0 °C of [14C]-amino acid-labelled virus which had adsorbed to cells for 60 min at 0 °C. Elution was carried out in medium (●●●●) and in medium containing 10 mM-EDTA (○○○○). Dissociated at 0 °C in medium containing 10 mM-EDTA (Fig. 2b), while elution in medium alone for 60 min at 0 °C dissociated less than 1% of the virus from the cells. Comparison of Fig. 2b with Fig. 1a suggests that virus adsorbed to cells at 0 °C remains in a complex similar to that formed after brief reaction between virus and cells at higher temperatures.

Lack of relationship between EDTA-mediated elution and cell-mediated eclipse

Virus particles of HRV-2 adsorbed to cells at 0 °C do not undergo detectable alteration to non-infectious subviral particles during 80 min of incubation (unpublished results). To test whether the ability of virus adsorbed at 0 °C to be eluted by EDTA was due to the block of eclipse at this temperature, [14C]-labelled HRV-2 was both adsorbed to cells and then eluted with EDTA in the presence of 10⁻⁴ M-SDS, which inhibits the cell-mediated formation of non-infectious particles (Lonberg-Holm & Noble-Harvey, 1973). Fig. 3 shows that SDS does not inhibit the normal progression of cell-associated virus from an elutable to a nonelutable form during 30 min of adsorption at 25 °C. The elution patterns after 1, 10, and 30 min of adsorption are statistically different as judged by the Mann-Whitney U test (Goldstein, 1964).

Other experiments tested the elution at 0 °C of virus adsorbed at 34.5 °C in the presence of 10⁻⁴ M-SDS. HRV-2 adsorbed 15 min at 34.5 °C, either in the presence or absence of SDS, eluted to the same small extent (2.5%) during 60 min of incubation in medium containing EDTA at 0 °C (data not shown). Thus, virus can progress from an EDTA-elutable to an EDTA-stable complex with the cell in the absence of eclipse.
Fig. 3. Elution of HRV-2 in medium containing EDTA and SDS following attachment in the presence of SDS. [14C]-amino acid-labelled HRV-2 was adsorbed to HeLa cells at a multiplicity of 10^4 particles/cell at 25 °C, in the presence of 30 µg/ml SDS, for 1 min (○—○), 10 min (□—□), and 30 min (△—△). The cells were washed in medium containing 30 µg/ml SDS, resuspended in medium containing 30 µg/ml SDS and 10 mM-EDTA, and samples were taken at various times after incubation to determine the percent of cell-associated [14C] (see Methods). The net portion of the original virus which became cell-associated was 94.4% (1563 ct/min) after 1 min of adsorption, 54.5% (6457 ct/min) after 10 min, and 81.2% (6419 ct/min) after 30 min. Fractions of each sample (cellular, supernatant, and wash) were counted for 20 min to calculate the percent of radioactivity which was cell-associated (see Methods).

The number of attachment sites per cell

An important feature of the interaction between HRV-2 and suspended HeLa cells is that the number of virus attachment sites appears to be reduced at 0 °C. Fig. 4a shows the adsorption of HRV-2 at 0 °C as a function of input multiplicity. This should be compared with Fig. 4b which shows the greater capacity of cells to adsorb virus at 34.5 °C. In the construction of these curves, the amount of cell-associated virus at each input multiplicity is measured after the adsorption reaction, when irreversible virus-cell complexes have been formed. Although there is not complete saturation in either experiment, it can be seen that cells at the lower temperature have roughly ½ the capacity for virus attachment of the cells at the higher temperature.

Fig. 4b also compares the ability of cells to adsorb virus in the presence and absence of 10^-4 m-SDS. The lack of effect of SDS indicates that eclipse of the adsorbed virus does not contribute to the shape of the adsorption isotherm, and hence that the deficiency in receptor activity of cells at 0 °C cannot be attributed to the lack of virus eclipse at 0 °C.

DISCUSSION

Attachment of HRV-2 virus particles to cells is inhibited by the addition of EDTA to the medium (Lonberg-Holm & Korant, 1972). This inhibition may be viewed as a result of an increase in the dissociation constant for the initial virus-receptor complex, rather than as an inhibition of the rate of interaction, since EDTA dissociates virus particles from cells shortly after adsorption at 25 °C (Fig. 1a) or after adsorption at 0 °C (Fig. 2b), and the eluted virus is infectious (Table 1). Divalent cations may participate directly in the initial inter-
action between virus and cell, or may be required for maintaining the virus particle or the cellular receptors in a conformation required for interaction.

The second step of interaction is the formation of a complex which cannot be dissociated by EDTA (Fig. 1 b). This is inhibited if adsorption is carried out at 0 °C (Fig. 2 b). The cell-associated virus at this second stage is still infectious, and virus adsorbed in the presence of 10⁻⁴ M-SDS, which blocks eclipse (Lonberg-Holm & Noble-Harvey, 1973), is not eluted by EDTA (Fig. 3).

The third step of infection by HRV-2 leads to the eclipse phase. Infectivity is lost by the formation of an altered virus component (Lonberg-Holm & Korant, 1972). This step is strongly inhibited by 10⁻⁴ M-SDS in the medium (Lonberg-Holm & Noble-Harvey, 1973). The elution of non-infectious HRV-2 particles from HeLa cells upon prolonged incubation at physiological temperature has also been found to be inhibited by SDS (unpublished results). It appears likely that the production of non-infectious particles which elute from
cells is a direct consequence of the production of non-infectious virus components which remain cell-associated. This was also suggested after an investigation of the elution of non-infectious particles of Coxsackie virus B3 (Crowell, Landau & Philipson, 1971).

In Fig. 1, it is evident that after 1 min of adsorption of HRV-2, no elution of virus in medium alone is detectable after a total of 31 min of incubation at 25 °C. However, after HRV-2 is adsorbed for 20 min, followed by elution for 10 min (a total of 30 min at 25 °C), approx. 5% of the adsorbed virus elutes spontaneously. It appears that the probability of spontaneous elution of HRV-2 may be greater when virus adsorbs to cells whose receptor sites are already partially occupied. Other explanations involving changes in the cell surface upon incubation or heterogeneity within the population of virus particles cannot be dismissed, however.

The inhibition at 0 °C of the formation of an EDTA-stable complex between HRV-2 and cells and the reduced number of receptor-sites at this temperature suggests the possibility that these temperature-dependent phenomena are related.

Such a relationship could be rationalized in terms of the fluid mosaic model of the plasma membrane (Singer & Nicolson, 1972), in which the membrane components are free to diffuse laterally in the lipid bilayer. Cooling of the membrane should increase the viscosity of the lipid and decrease the diffusion of membrane components (Frye & Edidin, 1970). The binding of virus particles to cells could involve multivalent receptors, and hence depend upon the lateral diffusion of receptor subunits. The greater rate of attachment and the greater number of apparent receptor sites at 34·5 °C than at 0 °C (Fig. 4) may then reflect the inability of receptor subunits to coalesce at the lower temperature, if detectable binding required more than one subunit. Similarly, progression to an EDTA-stable virus-cell complex may require the diffusion of additional components to the receptor site.

An alternate possibility is that after initial attachment, infectious virus particles are internalized by the cell, thus becoming insensitive to elution by EDTA. Mandel (1967) has presented evidence that poliovirus penetrates the host cell in infectious form before the cell-mediated alteration of the virus particles. Viropexis, for example, would be expected to be dependent upon temperature. This in itself would not explain the decreased number of apparent binding sites at 0 °C, unless receptor material was recycled from internalized virus particles to the cell surface.

Another explanation would require that the conformation of the virus receptors is temperature-dependent. At 0 °C only a fraction of the receptors might be able to bind virus; and these might also have a reduced binding affinity and be incapable of forming EDTA-insensitive complexes with virus.

In summary, two steps in the attachment of HRV-2 to cells prior to cell-mediated 'eclipse' can be experimentally resolved. Further work will be necessary to elucidate the molecular basis for these steps.

Jane Noble-Harvey was supported by the University of Delaware Research Foundation. We thank D. J. Reardon for technical assistance and Dr R. Z. Lockart, Jun., for encouragement and support.

REFERENCES


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*(Received 26 March 1974)*