The Role of Surface Sialic Acid in Adenovirus-cell Adsorption

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Recent studies on adenovirus adsorption have shown (Neurath, Hartzell & Rubin, 1969, 1970) that the erythocyte receptors for adenovirus differ from the receptors for other viruses hitherto described, e.g. influenza virus (Springer, Nagai & Tegtmeyer, 1966), in that arginine residues in the virus capsid and carboxylic acid groups of aspartic and glutamic acid residues of cellular proteinaceous components are involved in the interaction of adenovirus with cell membrane. Experiments of adenovirus attachment on to permissive cells suggest that the critical arginine residues are located in the vertex projections (Philipson, Lonberg-Holm & Petterson, 1968).

Although carbohydrates do not seem to participate in adenovirus-cell interaction, it is interesting to investigate the possible role of a carbohydrate moiety (especially of neuraminic acid residues) of plasma membrane glycoproteins on the adsorption of adenovirus type 2 to intact cells under conditions of a normal virus infection. A partial characterization of the complementary sites between adenovirus and cell receptors had resulted from experiments using red blood cells (RBC) or RBC ghosts as adsorption model systems. However these studies used denaturing reagents (Neurath, Hartzell & Rubin, 1970), which are impossible to utilize in an in vivo approach to the phenomenon. In this study, adsorption of labelled adenoviruses was explored after treatment of the host cells maintained in culture medium by glycosidases with a pH optimum in the neutral pH zone, preserving the cell viability.

Adenovirus type 2, a representative of Rosen's subgroup III (Rosen, 1960) was uniformly labelled in its proteins with [3H]-valine (1 mc/2 l. of KB cell culture in valine-deprived medium). The virus was extracted with fluorocarbon and purified in CsCl gradients according to conventional techniques (Green & Pifia, 1963). The specific activity of the virus preparations used was $10^6$ to $10^7$ counts/min./$10^9$ infecting cell units (ICU, Warocquier, Ménard & Samaille, 1966).

Three mammalian cell types were tested for adenovirus adsorption: (i) KB cells, (ii) adenovirus type 2-transformed rat embryo cells (Ad2-RE cells, strain 8617, originally obtained from Dr M. Green (Fujinaga & Green, 1970), and (iii) a tissue culture line of mouse fibroblastic cells (MF₂), originating from a mouse plasmocytoma, which continuously produce type C virus particles (this cell line was kindly supplied by Dr A. Paraf (Paraf et al. 1970). These three cell types have been adapted to suspension culture, and only KB cells have proved permissive for adenovirus (unpublished data). They have different sialic acid contents, as measured by the thiobarbituric acid method of Warren (1959) after hydrolysis of cells in 0.1 N-H₂SO₄ at 80°C for 1 hr. (Ohta, et al. 1968), viz. 1.35, 1.08 and 1.41 μmole of sialic acid per $10^9$ cells in KB, MF₂ and Ad2-RE cells, respectively. Approximately two-thirds of the total sialic acid is located at the cell surface (Kraemer, 1966; Glick et al. 1971).

Neuraminidase was extracted from Diplococcus pneumoniae and purified according to the method of Hughes & Jeanloz (1964). No proteolytic activity was detected in the final purified enzyme fraction. The specific activity of the enzyme preparations was 650 to 700 units/mg. protein (Lowry et al. 1951). One enzyme unit was defined as the amount of enzyme that releases 1 nmole of N-acetyl-neuraminic acid from the substrate ($\alpha$-acid glycoprotein.
from human serum) per min. at 37°, in 0.1 M-phosphate-citrate buffer pH 6.5. This enzyme specifically splits the N-acetyl-neuraminic acid linkages and is partly inhibited by Ca\(^{2+}\) and Mn\(^{2+}\) (Hughes & Jeanloz, 1964). To determine optimal enzyme concentrations, aliquots of cell culture were centrifuged, the cells washed with calcium-free phosphate buffered saline (PBS), pH 7.0, resuspended in PBS, and subjected to neuraminidase digestion by increasing amounts of enzyme preparation. After 15 min. incubation at 37°, the cells were centrifuged and released sialic acid was assayed in the supernatant fluid (Warren, 1959). All enzyme treatments of cells were carried out in the maximal sialic acid removal conditions, corresponding to 100 to 150 units/10\(^8\) cells.

Neuraminidase digestion of cells was performed as follows: cells were centrifuged at low speed at room temperature, gently resuspended in the usual adsorption medium (minimum Eagle's medium calcium-deprived and supplemented with 1% bovine foetal serum) at a concentration of 2 to 5 \(\times 10^6\) cells/ml., and maintained in suspension in spinner flasks at 37°. \textit{D. pneumoniae} neuraminidase (DPN) was added to the incubation medium (150 units/10\(^8\) cells). After incubation for 15 min. at 37°, CaCl\(_2\) was added up to a final concentration of 3 mM. A control culture was maintained in the same conditions without enzyme treatment.

\[^{3}H\]-valine-labelled adenovirus particles were added to DPN-treated and untreated cells, at an input multiplicity of 50 to 100 ICU/cell and adsorption was allowed to proceed for 45 min. at 37° with stirring. Cells were then centrifuged and washed three times in PBS to remove unadsorbed virus particles. The cell pellets were resuspended in an equal volume of PBS and dissolved in hyamine hydroxyde (2 vol. hyamine hydroxyde per vol. final cell suspension) by stirring overnight at 37°. The cell-adsorbed radioactivity was determined by counting samples of solutions thus obtained in Bray's scintillation fluid (Bray, 1960). In some experiments, CaCl\(_2\) was omitted and adsorption compared on control and DPN-treated cells.

The data obtained from neuraminidase treatment of KB, MF\(_2\) and Ad2-RE cells are summarized in Table I. After DPN digestion, the adenovirus adsorption by KB cells was significantly and reproducibly increased (19 to 38% over control). The difference in number of adsorbed adenovirus particles between DPN-treated and control KB cells was increased in the presence of Ca\(^{2+}\) (49 to 86% of additionally adsorbed radioactivity). The difference between DPN-treated and untreated cells was less pronounced (if any) for MF\(_2\) (6 to 17%) and Ad2-RE (4%) cells than for permissive KB cells. The increased number of adsorbed virus particles seemed dependent upon the multiplicity of infection in the inoculum. The percentage of additionally adsorbed virus particles was higher for lower multiplicities of infection (over 30% at 10 to 50 ICU/cell), whereas the percentage of additional adsorbed radioactivity was only about 10% at over 100 ICU/cell (Table 2).

At pH value of 7.0, both adenovirus particles and cell membrane have a net negative electric charge (Neurath \textit{et al.} 1970). The increase in adenovirus adsorption could be merely due to a decrease of the electrostatic repulsive forces between virus particles and cell membrane, after removal of the neuraminic acid residues. If this was the case, one would expect no significant variation in the number of adsorbed virus particles after further degradation of the carbohydrate chains of the cell membrane glycoproteins.

Since L-fucosyl residues are usually located on the non-reducing end of these carbohydrate chains, the neuraminidase-treated cells were subsequently submitted to the action of an \(\alpha\)-L-fucosidase with a pH optimum of 6.0 but which was still active at neutral pH. The enzyme used could act on glycoproteins and was an \(\alpha\)-(1 \(\rightarrow\) 2)-specific L-fucosidase, extracted from \textit{Clostridium perfringens} and purified according to Aminoff \& Furukawa (1970). The enzyme preparation was purified 20-fold and was cleared of neuraminidase, hexosaminidase and protease activities. Its specific activity was 45 units/mg. protein. One enzyme unit was
### Table 1. Comparative adsorption of labelled adenovirus on untreated and Diplococcus pneumoniae neuraminidase (DPN)-treated mammalian cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Total radioactivity in inoculum (counts/min.)</th>
<th>Untreated cells (u)</th>
<th>DPN-treated cells (n)</th>
<th>Ratio, n:u</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>KB</td>
<td>80,000</td>
<td>6,538</td>
<td>9,054</td>
<td>1.38</td>
</tr>
<tr>
<td>2*</td>
<td>KB</td>
<td>80,000</td>
<td>3,462</td>
<td>4,119</td>
<td>1.19</td>
</tr>
<tr>
<td>3</td>
<td>KB</td>
<td>80,000</td>
<td>8,542</td>
<td>13,200</td>
<td>1.55</td>
</tr>
<tr>
<td>4</td>
<td>KB</td>
<td>80,000</td>
<td>11,453</td>
<td>17,107</td>
<td>1.49</td>
</tr>
<tr>
<td>5</td>
<td>KB</td>
<td>100,000</td>
<td>7,818</td>
<td>12,354</td>
<td>1.58</td>
</tr>
<tr>
<td>6</td>
<td>KB</td>
<td>250,000</td>
<td>21,368</td>
<td>39,449</td>
<td>1.86</td>
</tr>
<tr>
<td>7</td>
<td>MF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>100,000</td>
<td>6,490</td>
<td>7,134</td>
<td>1.10</td>
</tr>
<tr>
<td>8</td>
<td>MF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>100,000</td>
<td>6,882</td>
<td>8,053</td>
<td>1.17</td>
</tr>
<tr>
<td>9</td>
<td>MF&lt;sub&gt;3&lt;/sub&gt;</td>
<td>400,000</td>
<td>40,774</td>
<td>43,099</td>
<td>1.06</td>
</tr>
<tr>
<td>10</td>
<td>Ad2-RE</td>
<td>400,000</td>
<td>14,869</td>
<td>15,473</td>
<td>1.04</td>
</tr>
<tr>
<td>11</td>
<td>Ad2-RE</td>
<td>400,000</td>
<td>11,547</td>
<td>12,083</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Cells were maintained in spinner culture at a concentration of 2 to 5 x 10<sup>6</sup> cells/ml. Total cell number per spinner flask was 2 x 10<sup>8</sup> cells. [aH]-valine-labelled adenovirus was inoculated at a multiplicity of 50 to 100 infecting cell units/cell.

* Calcium omitted in control and neuraminidase-treated cells.

### Table 2. Relationship between multiplicity of infection and adsorption on untreated and Diplococcus pneumoniae neuraminidase (DPN)-treated KB cells

<table>
<thead>
<tr>
<th>Input multiplicity (ICU*/cell)</th>
<th>Adsorbed radioactivity (counts/min.)</th>
<th>Untreated cells (u)</th>
<th>DPN-treated cells (n)</th>
<th>Ratio, n:u</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>14,863</td>
<td>19,700</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>35,984</td>
<td>49,199</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>55,810</td>
<td>64,293</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>70,083</td>
<td>78,337</td>
<td>1.11</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>82,290</td>
<td>90,917</td>
<td>1.06</td>
<td></td>
</tr>
</tbody>
</table>

Untreated and DPN-treated KB cells were maintained in suspension culture at a concentration of 2 x 10<sup>6</sup> cells/ml.

* ICU: infecting cell unit.

Defined as the amount of enzyme that released 1 n mole of fucose per min. from a purified glycoprotein with blood group H-specificity, isolated from ovarian cyst fluid, at 37 ° in 0.26 M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01 M-CaCl<sub>2</sub> buffer pH 6.5. In order to determine the enzyme amount required to produce a maximum release of fucose, KB cells were prior incubated in PBS for 15 min. with neuraminidase and for a further 15 min. with increasing amounts of enzyme preparation, incubation mixtures centrifuged, and released fucose, sialic acid and amino-sugars determined in the supernatant fluid. L-Fucose content was determined, after enzymatic conversion to L-fuculose (Green & Cohen, 1956) by an adaptive isomerase extracted from a mutant strain of Escherichia coli (E. coli Ba15, kindly supplied by Dr S. Cohen, University of Pennsylvania), by the cysteine-carbazole reaction (Dische & Borenfreund, 1951). A maximum fucose release was reached with 150 to 200 units/2 x 10<sup>6</sup> cells. No N-acetyl-hexosamine was released from the cell membrane, as determined by the method of Reissig, Strominger & Leloir (1955).

KB cell suspension cultures were incubated in parallel in adsorption medium at 37 °. One culture was untreated and served as control, the others were treated respectively with DPN,
Table 3. Comparative adsorption of labelled adenovirus on untreated, Diplococcus pneumoniae neuraminidase (DPN), and Clostridium perfringens fucosidase (CPF)-treated KB cells

<table>
<thead>
<tr>
<th>KB cells</th>
<th>Radioactivity adsorbed (counts/min.)</th>
<th>Ratio versus untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8,285</td>
<td>1.00</td>
</tr>
<tr>
<td>DPN-treated</td>
<td>12,845</td>
<td>1.55</td>
</tr>
<tr>
<td>Heat-inactivated DPN</td>
<td>8,350</td>
<td>1.00</td>
</tr>
<tr>
<td>CPF-treated</td>
<td>9,030</td>
<td>1.09</td>
</tr>
<tr>
<td>Heat-inactivated CPF</td>
<td>9,157</td>
<td>1.10</td>
</tr>
<tr>
<td>DPN + CPF</td>
<td>12,524</td>
<td>1.51</td>
</tr>
<tr>
<td>DPN + CPE*</td>
<td>31,558</td>
<td>3.80</td>
</tr>
</tbody>
</table>

* CPE corresponds to the fraction obtained at the first purification step of the fucosidase from Clostridium perfringens, i.e. ammonium sulphate precipitate.

heat-inactivated DPN, C. perfringens fucosidase (CPF), heat-inactivated CPF, DPN followed by CPF (15 min. incubation each) and DPN followed by a further incubation with a partly purified extract from C. perfringens (CPF). CPE corresponded to the ammonium sulphate fraction obtained at the first purification step, and contained, besides fucosidase, a number of exo- and endoglycosidases (Aminoff & Furukawa, 1970) and also a minor proteolytic activity. At the end of the incubation period, aliquots of labelled adenovirus suspension corresponding to a multiplicity of 50 ICU/cell were added to the cultures and adsorption conducted at 37° for 45 min. Cell-adsorbed radioactivity was determined as above-mentioned and compared in the different cell cultures. Table 3 demonstrates that DPN + CPF-treated KB cells did not adsorb more virus than did DPN-treated cells. After DPN + CPE treatment, the quantity of adsorbed adenovirus was almost four times higher than on control cells, and 2.5 times higher than on neuraminidase-treated cells.

The removal of sialic residues from the cell surface therefore increased the capacity of cell to attach adenovirus. A possible role of proteolytic enzymes in this process can be ruled out since no proteolytic activity was detectable in the neuraminidase preparations used. In addition it has been shown that the proteolytic enzymes destroy the erythrocyte receptors for adenovirus (Neurath et al. 1969). In contrast it was found that the receptors of permissive cells were only destroyed by proteolytic enzymes with a broad specificity (pronase or subtilisin) and that the attachment rate for adenovirus was enhanced by treatment of intact cells with trypsin and chymotrypsin. However, these mild proteolytic digestions did not change the adsorption isotherm and consequently the final total adsorbed adenovirus, indicating that trypsin nor chymotrypsin treatment did not expose additional binding sites for the virus (Philipson et al. 1968). Presence of some protease in the crude extract from C. perfringens cannot therefore explain the increased capacity of cells to adsorb the virus.

The possible role of neuraminic acid residues in adenovirus red cell-adsorption has been investigated after treatment of RBC ghosts with Vibrio cholerae neuraminidase and subsequent determination of the haemagglutination-inhibiting titre (Neurath et al. 1969). This method was unable to detect an increased adsorption of adenovirus haemagglutinin after neuraminidase digestion. The results obtained by previous workers (Philipson et al. 1968; Neurath et al. 1970) demonstrating the proteinaceous nature of adenovirus cell-receptors seemed to exclude the participation of carbohydrates in the receptors. This latter point has to be reconsidered in the light of the data herein presented.

It has been shown recently that the removal of carbohydrates from cell surfaces by
glycosidases results in alterations of cellular interactions (Woodruff & Gesner, 1967; Kemp, 1968; Buck, Glick & Warren, 1970). Moreover, recent experiments (Ray & Simmons, 1971) have clearly demonstrated an increased susceptibility to antibody-mediated lysis in neuraminidase-treated cells. That removal of neuraminic acid residues results in an increased adenovirus adsorption suggests that the carbohydrate moiety of cell membrane glycoproteins might be also implicated in the adenovirus adsorption phenomenon. This increased adenovirus-permissive cell interactions can be due to different mechanisms. (i) The sialic acid residues removed by neuraminidase from the cell membrane may cause steric hindrance between adenovirus fibres and cell receptors. (ii) Neuraminidase also reduces the negative charge on the cell surface, thus decreasing the repellent electrostatic forces between virus particle and host cell. (iii) Reducing the surface negative charge by removing the mutually repulsive negative charges of the sialic acid residues, thereby reduces the rigidity of the cell surface (Weiss, 1965); the increased deformability of the cell membrane may favour the contact between adenovirus particles and cell receptors. All of these processes are probably involved in the increased adsorption of adenovirus on KB cell.

The first mechanism invoking a steric hindrance to the adenovirus adsorption cannot be excluded from the experimental findings that splitting the fucosidic residues after removal of the sialic acid did not increase virus adsorption. First, because fucose removal can be insufficient to permit the detection of a significant difference in adsorption, and secondly, because incubation of neuraminidase-treated KB cells with a C. perfringens extract containing endoglycosidases, that is shortening the carbohydrate side chains of membrane glycoproteins, results in substantial increase of virus adsorption, presumably due to a better exposure of proteinaceous sites which bind adenovirus. The second hypothetical mechanism, postulating a decrease in repulsive charges between adenovirus and host cell is supported by the finding that Ca\(^{2+}\) facilitates adenovirus adsorption, probably by neutralizing the negative charge of the cell surface. Although it has been demonstrated (Weed, Lacelle & Merril, 1969; Forstner & Manery, 1971) that sialic acid anions have a lower affinity for Ca\(^{2+}\) than the amino acid carboxyl groups, because of its low pK of 2.6 (Eylar et al. 1962), and its terminal position on glycanic side chains of glycoproteins, sialic acid undoubtedly binds Ca\(^{2+}\) cations (Forstner & Manery, 1971). No significant difference was observed in adenovirus adsorption between untreated and neuraminidase-treated MF\(_2\) and Ad\(_2\)-RE cells: this would imply that adsorption which occurs in the non-permissive cells could be a non-specific phenomenon.

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REFERENCES


Short communications


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