Neuraminidase-sensitive Erythrocyte Receptor for Enterovirus Type 70

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SUMMARY

Enterovirus type 70 (EV70) agglutinated human ‘O’ erythrocytes at 4 °C as well as 22 °C, but visible agglutination was lost when warmed at 37 °C although the virus remained attached to the surface of the erythrocyte. The receptor sites for the virus were neuraminidase-sensitive. A direct involvement of sialic acid on the cell surface in virus–cell interaction was confirmed by the fact that the presence of fetuin or free N-acetylneuraminic acid inhibited the haemagglutinating activity of EV70. Similar numbers of virus particles were required for 1 haemagglutinating unit (HAU) of EV70 and 1 HAU of mengovirus, whereas 2-6-fold or more of virus particles of echovirus type 7 and type 11 gave the same activity. On the other hand, the number of receptor sites on the cell surface for EV70 was found to be sevenfold more than for mengovirus. Therefore, the erythrocyte receptor for EV70 is different from that for common enteroviruses and similar, though not identical, to the cardiovirus receptor. However, serological tests such as neutralization, complement fixation or haemagglutination inhibition did not reveal any common antigen between EV70 and cardiovirus.

INTRODUCTION

Enterovirus type 70 (EV70), a causative agent of acute haemorrhagic conjunctivitis, was first isolated in 1971 (Kono et al., 1972) and on the basis of its physicochemical properties, the virus was eventually classified as a member of the genus Enterovirus (Mirkovic et al., 1973). However, some differences compared to other common enteroviruses were noticed in its biological properties. (i) The primary site of the virus replication in vivo is in the conjunctiva and not in the alimentary tract, unlike ordinary enteroviruses, since the virus is seldom isolated from faeces. (ii) EV70 adsorbs to and replicates in a wide variety of non-primate cell cultures including rabbit, cow, pig, mouse and hamster tissues (Yoshii et al., 1977). In contrast, most other enteroviruses multiply only in primate cells. These facts suggest that the cell receptor site for EV70 is different from those for other enteroviruses.

In this communication, we describe the results of a study comparing EV70 to other picornaviruses in virus–erythrocyte interactions and try to explain the unique features of EV70 in this respect. The haemagglutinating activity of EV70 was described previously (Kono et al., 1978). Two haemagglutinating enteroviruses, echovirus type 7 (E7) and type 11 (E11), as well as two cardioviruses, mengovirus and encephalomyocarditis virus (EMCV), were selected as reference viruses to compare their interaction with erythrocytes.

METHODS

Virus and haemagglutinin (HA). The prototype strain of EV70 (J670/71), the Wallace strain of E7 and the YC-71-106 strain of E11 isolated in our laboratory in 1971, were propagated in LLC-MK2 cells. The incubation temperature was 33 °C for EV70 and 37 °C for the echoviruses. EMCV and mengovirus were propagated in BHK-21 cells at 37 °C. All viruses were purified by the method previously described by Kono et al. (1978). The virus was precipitated from the culture fluid by stirring with 8% polyethylene glycol 6000 (Wako, Osaka, Japan) and 0·4 M-NaCl. The pellet was collected and, after resuspension in 1/50 the original volume, CsCl was added at a starting density of 1·33 g/ml. The solution was centrifuged in a Beckman SW50.1 rotor at 35000 rev/min for 18 h at 4 °C.
Virus fractions with peak HA activity were collected and dialysed against phosphate-buffered saline (PBS) and employed as the source of HA antigen.

**Erythrocytes.** Human group 'O' blood was collected in Alsever solution. Cell concentration was determined photometrically according to the method of Hierholzer & Suggs (1969).

**HA test.** The HA test was performed in a microtitre system (Cooke Engineering Co., Alexandria, Va., U.S.A.) using human 'O' erythrocytes. Serial twofold dilutions of HA antigen were prepared in 50 μl vol. (25 μl virus + 25 μl diluent) in wells of 'U' plates, and an equal volume of 0-4% human 'O' erythrocyte suspension was added to each well. The plates were shaken and the erythrocytes were allowed to settle at 4 °C for 18 h. The highest dilution of antigen which showed complete haemagglutination was considered as the endpoint and this concentration was defined as 1 haemagglutinating unit (HAU). The diluent used was PBS (pH 7-6) containing 0-0005% gelatin and 0-005% bovine serum albumin fraction V.

**Treatment of human 'O' erythrocytes with neuraminidase.** A 10% erythrocyte suspension was mixed with an equal volume of PBS containing various concentrations of neuraminidase (Sigma). After 1 h incubation at 37 °C, cells were washed twice with cold PBS, resuspended in PBS at the original concentration and assayed for ability to adsorb viruses.

**Electron microscopy.** A suspension of polystyrene latex beads (Dow Chemical Co., Midland, Mich., U.S.A.; 109 nm diam. and 1.42 × 10^{14} particles/ml in 10% bovine serum albumin solution) was added to the HA preparation at a concentration of 2.84 × 10^{11} latex particles/ml. The mixture was combined with an equal volume of 3% phosphotungstic acid and applied to collodion-coated 300-mesh copper grids. Virus particles and latex spheres on the grids were photographed in a JEM-100CX electron microscope. The virus particle concentration was calculated from the virus latex particle ratio.

**Serological tests.** The neutralizing (NT) antibody titres were expressed as reciprocals of the highest dilution giving plaque reduction of 50% or more. To assay for EV70, LLC-MK2 cells were used and overlaid with Eagle's minimal essential medium containing 0-1% bovine serum albumin and 0-9% Bactoagar. For EMCV or mengovirus, BHK-21 cells were infected and 0.9% agarose was employed instead of Bactoagar. Haemagglutination inhibition (HI) tests were carried out as reported previously (Kono et al., 1978). Complement fixation (CF) tests were performed in microplates using five 50% haemolytic units of complement.

**Antisera.** Anti-EMCV guinea-pig serum was kindly given by Dr F. Brown, Animal Virus Research Institute, Pirbright, Woking, Surrey, U.K. The hyperimmune monkey serum against the J670/71 strain used for plaque reduction assay has been described previously (Sasagawa et al., 1982). Anti-EV70 serum used for HI and CF tests was prepared by inoculating a guinea-pig with a purified preparation of J670/71 virions; the virus was concentrated and banded in CsCl as described above, and further purified by zonal centrifugation through sucrose gradients. The purified virion fraction obtained was mixed with an equal volume of Freund's complete adjuvant and the animal received a total of 0-4 ml mixture by footpad route. One month later the animal was twice inoculated intraperitoneally with 0-4 ml aqueous antigen with an interval of 1 week, and bled 1 week later.

**RESULTS**

**Attachment of EV70 on to the human 'O' erythrocyte**

Attachment of the virus particles to human 'O' erythrocytes was measured by assaying residual HA in supernatants after allowing virus to interact with erythrocytes. Equal volumes of a virus HA antigen and a serially diluted erythrocyte suspension were mixed. After incubating for 1 h at the indicated temperature, the cells were removed by low-speed centrifugation (at the incubation temperature) and HA present in the supernatant was titrated.

As shown in Fig. 1, HA titres in the supernatants decreased linearly at 0 °C, 22 °C or 37 °C. Despite the absence of a visible haemagglutinating pattern of EV70 at 37 °C, HA depletion was observed in the supernatant in proportion to the amount of erythrocytes added. Since inactivation of HA did not occur under these conditions (data not shown), the decrease of HA in the supernatant was considered to be due to the attachment of virus to erythrocytes. At 37 °C, however, unbound HA in the supernatant appeared to increase, suggesting that binding of the virion to the erythrocyte became unstable at higher temperature. Similar linear adsorption of EV70 by erythrocytes was also observed by titrating the virus infectivity in the supernatant (data not shown).

**Comparison of efficiency of adsorption on to erythrocytes of EV70 and other picornaviruses**

Adopting the same experimental procedure as in Fig. 1, 1280 HAU of purified E7, E11, EV70 and mengovirus were mixed with an equal volume of twofold serial dilutions of erythrocyte
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Fig. 1. Attachment of EV70 on to human 'O' erythrocytes at 0 °C (●), 22 °C (△) and 37 °C (▲). A 0.2 ml amount of EV70 antigen containing 1280 HAU was mixed with an equal volume of twofold serially diluted erythrocyte suspension and incubated for 1 h at the indicated temperature. The erythrocytes were removed by centrifugation at 2500 rev/min for 5 min and the amount of virus in the supernatant was determined by HA titration as described in Methods.

Fig. 2. Comparison of efficiency of adsorption on to erythrocytes of EV70 and other picornaviruses. The method used is the same as that described in the legend to Fig. 1 except for the four kinds of viruses used as antigens and 0 °C being the only temperature employed. ●, EV70; △, mengovirus; ○, E11; ▲, E7.

Effect of temperature shift-up on the virus–erythrocyte complex

Preparations of four different picornaviruses (EV70, E7, E11 and mengovirus), adjusted to contain 128 HAU, were adsorbed on to erythrocytes at 0 °C and then the incubation temperature was shifted to 37 °C. Samples were taken at every hour from 0 to 4 h and then at 24 h to examine patterns of erythrocyte sedimentation. HA released in the supernatant was then titrated after pelleting cells at 2500 rev/min for 5 min (Fig. 3).

In the case of E7 and mengovirus HA, the pattern of erythrocyte sedimentation was not affected by temperature increase and no HA was detected in the supernatant. The E11 eluted from erythrocytes at 37 °C causing loss of haemagglutination. In contrast, elution of EV70 HA was not observed in the supernatant at 37 °C despite disappearance of haemagglutination. The possibility that EV70 loses haemagglutinating activity during the process of attachment and release was excluded by the following experiment. EV70 HA was titrated on a microplate at 0 °C and the microplate was then warmed to 37 °C, at which temperature the agglutination previously formed at 0 °C diminished immediately. Subsequently, the plate was agitated and cooled to 0 °C, and the original agglutination was restored. This proved that EV70 HA was not inactivated but remained on the cell surface throughout the experiment, even after haemagglutination was diminished at 37 °C.

Effect of neuraminidase treatment of the erythrocyte

Human 'O' erythrocytes were treated with various concentrations of neuraminidase as described in Methods. The levels of HA were adjusted to 64 HAU for each virus, and these were
Fig. 3. Effect of temperature on virus–erythrocyte interaction. Test tubes containing 0·2 ml amounts of HA antigens and an equal volume of a 10% erythrocyte suspension were kept at 0 °C in an ice-bath for 1 h, after which tubes were warmed to 37 °C in a water-bath. One tube of each antigen was taken out at the indicated time after transfer, haemagglutination was read and the supernatant fluid was collected by centrifugation to titrate released HA. O, EV70; △, E7; ▲, mengovirus; □, E11. (+), haemagglutination-positive; (−), haemagglutination-negative.

Fig. 4. Effect of neuraminidase pretreatment of erythrocytes on ability to adsorb to viruses. Human ‘O’ erythrocytes were mixed with the indicated concentration of neuraminidase and treated as described in Methods. To 0·2 ml samples of antigen containing 64 HAU, an equal volume of a 10% suspension of the treated erythrocytes was added, and after 1 h at 0 °C, HA reduction of the supernatants was assayed. ▲, mengovirus; O, EV70; △, E7; O, E11.

mixed with equal volumes of suspensions of neuraminidase-treated erythrocytes. After 1 h at 0 °C, free HA remaining in the supernatant was titrated. Untreated cells were included as controls. Fig. 4 shows the results of the experiment. Neuraminidase treatment severely impaired the ability of the cells to bind EV70 and mengovirus; treatment with 2·5 mU/ml of neuraminidase eliminated the ability of the cells to adsorb these two viruses. In contrast, the binding capacity of cells with E7 and E11 viruses remained unchanged as compared with untreated cells, even after treatment with 40 mU/ml of the enzyme, as was expected from the evidence of a previous report (Podoplekin, 1964) and also from general experience with human enteroviruses.

Inhibition of virus HA activity with fetuin or N-acetylneuraminic acid (NANA)

Neuraminidase induces release of terminal sialic acid from sialoglycoproteins. If such terminal sialic acid residues on cell surfaces are directly involved in the attachment of EV70 or mengovirus, either free NANA or a substrate of the enzyme such as fetuin should be accessible to the surface of the viruses, as a result inhibiting the attachment of virus to the cell. To test this, viruses were mixed with various concentrations of fetuin (Sigma) or NANA (Sigma) in microplate wells. Duplicate plates were prepared, and one was incubated at 37 °C and the other was kept at 4 °C. After 1 h an equal volume of 0·4% erythrocyte suspension was added to the wells and the agglutination in each well was examined after 18 h at 4 °C (Fig. 5). Fetuin concentrations of up to 10 mg/ml had no inhibitory effect on the HA activity of E11, whereas 5 mg/ml inhibited E7 HA activity. Mengovirus lost its HA activity gradually with increasing fetuin concentration up to 2·5 mg/ml, whereas 0·625 mg/ml rendered 32 units of EV70 HA completely non-agglutinable. Similar results were obtained with free NANA solution: NANA concentration up to 1·25 mg/ml had no effect on E11 and E7, while it slightly impaired mengovirus HA and EV70 HA activity was lost completely at 2·5 mg/ml. The inhibitory effect of
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Fig. 5. Inhibition of virus HA activity with fetuin or NANA. Serial twofold dilution of HA antigens were made in wells of duplicate microplates and mixed with an equal volume (25 μl) at the indicated concentration of fetuin (a) or NANA (b). The plates were incubated at 37 °C for 1 h, after which a 0·4% erythrocyte suspension was added. The haemagglutination result was read after being kept at 4 °C overnight. ●, EV70; △, mengovirus; △, E7; ○, E11.

Table 1. Correlation of HA and virus particle counts

<table>
<thead>
<tr>
<th>Virus</th>
<th>HA units (× 10^3)</th>
<th>No. of particles/ml (× 10^11)</th>
<th>No. of particles/1 HAU (× 10^8)</th>
<th>No. of particles/cell for 1 HAU</th>
<th>Saturating no. of particles/cell</th>
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</thead>
<tbody>
<tr>
<td>EV70</td>
<td>3·2</td>
<td>16·3 ± 5·7</td>
<td>5·1</td>
<td>16·8</td>
<td>2688</td>
</tr>
<tr>
<td>E7</td>
<td>6·4</td>
<td>84·8 ± 32·5</td>
<td>13·3</td>
<td>43·8</td>
<td>219</td>
</tr>
<tr>
<td>E11</td>
<td>6·4</td>
<td>136·7 ± 61·0</td>
<td>21·4</td>
<td>70·4</td>
<td>704</td>
</tr>
<tr>
<td>Mengovirus</td>
<td>3·2</td>
<td>18·0 ± 8·8</td>
<td>5·6</td>
<td>18·4</td>
<td>368</td>
</tr>
</tbody>
</table>

fetuin was not dependent on the temperature at which it was mixed with virus antigen before addition of erythrocytes, while NANA inhibited HA of two viruses only when the mixture was incubated at 37 °C. The results indicated that sialic acid was actually involved in the attachment of the erythrocyte.

Particle counts and relation to HA activity

Since the concentration of virus particles in the undiluted preparation of purified virus antigens was calculated as described in Methods, and as the number of erythrocytes/ml was known from direct counting with a haemocytometer, it was possible to calculate the number of virus particles/cell at the level of 1 HAU (see Table 1). To calculate the number of particles in the well registering 1 HAU as shown in Table 1, the following dilution factors were taken into account: 100 μl of a final mixture in a well contained 50 μl of serially diluted virus and 50 μl of 0·4% erythrocyte suspension (3·04 × 10^7 cells/ml).

One HAU of EV70 required 17 virus particles/cell. Mengovirus required a similar number of virus particles/cell (18) for 1 HAU, while for 1 HAU E7 and E11 needed 44 and 70 virus particles/cell respectively (i.e. 2·6 times and 4·1 times greater respectively than EV70). The number of mengovirus particles required for 1 HAU was less than reported for EMCV by Angel & Burness (1977), i.e. 18 versus 200. The difference may be due to differences in the methods of virion counting, since they calculated from u.v. absorbance of virion preparation and particle weight of EMCV.

In order to estimate the number of virus particles required to saturate the receptor sites on erythrocytes, the number of particles/cell for 1 HAU (shown in column 4 of Table 1) was
Table 2. Antigenic relationship between EV70 and cardioviruses

<table>
<thead>
<tr>
<th>Test</th>
<th>Antiserum</th>
<th>Challenge virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EV70</td>
<td>Mengovirus</td>
</tr>
<tr>
<td>NT*</td>
<td>Anti-EV70</td>
<td>20480</td>
</tr>
<tr>
<td></td>
<td>Anti-EMCV</td>
<td>40</td>
</tr>
<tr>
<td>HI†</td>
<td>Anti-EV70</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>Anti-EMCV</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>CF†</td>
<td>Anti-EV70</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Anti-EMCV</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

* Reciprocals of serum dilution that caused a 50% plaque reduction.
† Reciprocals of endpoint.

multiplied by the HAU obtained in the experiment shown in Fig. 2. As many as 2688 EV70 virus particles were found to attach to a cell, whereas the maximum number of virions attaching per cell was 368, 219 and 704 for mengovirus, E7 and E11 respectively. Thus, it is obvious that the receptor sites for EV70 on human 'O' erythrocytes must be far more abundant than those for echoviruses and for mengovirus, although the latter reacts with a neuraminidase-sensitive cell receptor in common with EV70.

Antigenic relationship between EV70 and cardioviruses

In view of the experimental results indicating that the erythrocyte receptor for EV70 was similar to that for cardiovirus, the immunological relationship between these two was explored by NT, HI and CF tests as described in Methods. However, no cross-reactivity was revealed by any of these tests (Table 2).

DISCUSSION

Our comparative study showed that EV70 differs from other enteroviruses in its interaction with the receptors on erythrocyte surfaces.

First, it was shown that EV70 attached to erythrocytes and agglutinated them equally well at both 0 °C and 22 °C, but the agglutination was lost at 37 °C, the temperature at which the virus was still capable of attaching to the cell surface in proportion to its concentration. Erythrocytes which had been once agglutinated by EV70 at 0 °C were easily dispersed when the incubation temperature was elevated to 37 °C, but HA was not released in the supernatant. This feature was unique to EV70 and was not observed with the other picornaviruses studied. It has been reported that the effect of temperature on echovirus HA activity is quite variable even within one serotype (Podoplekin & Ivanova, 1965). Three isolates of EV70 which had been obtained in different areas and in different years revealed the same HA characteristics as the prototype.

Second, the receptor site of EV70 on the cell surface was found to be sensitive to neuraminidase. The same property was reported for cardiovirus (Verlinde & de Baan, 1949; Angel & Burness, 1977) and rhinovirus type 5 (Stott & Killington, 1972) but there are no reports of sensitivity to neuraminidase of enterovirus attachment to erythrocytes. Thus, EV70 HA resembles that of cardiovirus or rhinovirus rather than that of other enteroviruses. EV70 was reported to have another similarity to rhinovirus in growth characteristics: both viruses have a naturally occurring temperature-sensitive phenotype (Miyamura et al., 1974). However, there is a distinct difference between the two viruses in respect to acid stability and buoyant density in CsCl (Yamazaki et al., 1974).

The failure of certain viruses to attach to cells treated with neuraminidase is generally considered as evidence of a direct attachment of such viruses to sialic acid residues on the cell surface. However, it is also considered that removal of terminal sialic acid residues may cause the cell surface membrane to undergo conformational changes which result in the masking of the virus receptor on the surface. One of our experimental results excluded the latter possibility. The presence of free NANA or fetuin in the reaction mixtures had a severe inhibitory effect on the HA activity of EV70 and a moderate one on that of mengovirus. Angel & Burness (1977) showed
that free NANA had no inhibitory effect on haemagglutination of sheep cells by EMCV at a concentration of 1 mg/ml. Under the conditions used by us, NANA inhibited mengovirus HA activity at concentrations of 1-25 mg/ml or greater, but inhibition was observed only when the virus was incubated with NANA at 37 °C before addition of erythrocytes and not at 4 °C.

Third, our experiments revealed that EV70 was quantitatively differentiated from other viruses in its interaction with human 'O' erythrocytes as follows: (i) 1 HAU of EV70 and mengovirus required more or less equal numbers of virions, whereas 1 HAU of other echoviruses required 2-6 times or more; (ii) the number of EV70 particles required to saturate receptor sites on the erythrocyte surface was approximately 7 times greater than that of mengovirus.

Based on these observations, it is considered that the critical sites for virus-erythrocyte interaction are somewhat similar but not identical between EV70 and cardiovirus, and that a fine difference may exist in the capsid surface of these two viruses. It was reported that cardiovirus was degraded when exposed to mild acid in the presence of 0-1 M-chloride or -bromide (Mak et al., 1970), or somewhat unstable in 0-1 M-acetate at pH 3 (Newman et al., 1973). Our preliminary experiment revealed that EV70 was not inactivated by incubation at 37 °C for 60 min in a medium containing 0-1 M-chloride at pH 6 (Yamazaki et al., 1974), and thus could be differentiated from cardiovirus. Furthermore, antigenic relationships between EV70 and cardioviruses were not demonstrated by either NT, CF or HI tests. Nevertheless, it is considered that EV70 is related to, although not identical to, cardiovirus rather than to enterovirus as far as their receptor sites on the erythrocyte are concerned. Since cardioviruses are isolated from, and are pathogenic to, rodents it is speculated that EV70 might be derived from an animal virus. In fact, EV70 neutralizing immunoglobulin was found widely distributed in sera from many kinds of animals (Kono et al., 1981; Sasagawa et al., 1982). Thus, it would be interesting to know if there is any other animal picornavirus with which EV70 shares these receptor specificity or other growth characteristics in nature.

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