Attachment of Influenza C Virus to Human Erythrocytes

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(Accepted 6 July 1988)

SUMMARY

Binding experiments with radioactively labelled influenza C virions were carried out
in order to investigate the interaction of the virus with human erythrocytes. The erythrocytes
from any of 35 different individuals were found to contain influenza C virus-binding
sites though their number was variable among the individuals and was much less than
that on mouse, rat and chicken erythrocytes. Attachment of influenza C virus to human
erythrocytes was inhibited completely by prior treatment of the virus with anti-HE
monoclonal antibody having a strong haemagglutination inhibition activity. Pretreat-
ment of erythrocytes with neuraminidase or the neuraminase-O-acetyesterase of
influenza C virus resulted in a marked reduction in the level of virus binding. Thus it
appears that human erythrocytes have a low level of O-acetylated sialic acid-containing
glycoconjugates that can interact specifically with the HE glycoprotein of influenza C
virus. Proteolytic digestion of erythrocytes with ficin, bromelain or V-8 protease
inhibited virus binding almost completely, suggesting that the erythrocyte receptor for
influenza C virus is a glycoprotein. In contrast to these enzymes, trypsin treatment of
erythrocytes reduced virus binding by only about 50%, and α-chymotrypsin treatment
did not inhibit at all. It was also found that treatment of erythrocytes with monoclonal
antibody to the M or N blood group antigen greatly inhibited virus binding to the cells.
These results, taken together, suggest that most influenza C virus receptors on human
erythrocytes, if not all, reside on glycophorin A which is known to possess the M or N
blood group activity.

INTRODUCTION

In contrast to influenza A and B viruses which contain two distinct glycoproteins,
haemagglutinin and neuraminidase, influenza C virions have only a single type of glycoprotein
on their surface (Herrler et al., 1979, 1981; Sugawara et al., 1981). This glycoprotein, recently
designated HE (Vlasak et al., 1987), possesses both receptor-binding and receptor-destroying
activities (Sugawara et al., 1985; Vlasak et al., 1987). The nature of the cellular receptor for
influenza C virus as well as of the receptor-destroying enzyme (RDE) of the virus remained
unclear for a long time. It has been shown recently, however, that influenza C virus-specific
inhibitors in rat serum lost their haemagglutination inhibition activity after treatment with
neuraminidase or with alkali under mild conditions, and that neuraminidase treatment of
chicken erythrocytes prevented their agglutination by this virus (Herrler et al., 1985a, b; Kitame
et al., 1985). This suggests that alkali-labile sialic acid is an essential component of the influenza
C virus receptor. Furthermore, Herrler et al. (1985c) presented evidence that the RDE of the
virus is neuraminate-O-acetyesterase which releases an acetyl residue from position C-9 of 9-O-
acetyl-N-acetylanuraminic acid (Neu5,9Ac₂). The importance of Neu5,9Ac₂ as a receptor
determinant for influenza C virus was confirmed by Rogers et al. (1986), who demonstrated that
human erythrocytes enzymically modified to contain Neu5,9Ac₂ are agglutinated by the virus
whereas cells containing N-acetylanuraminic acid (Neu5Ac) or N-glycolylnearaminic acid
(Neu5G1) are not. However, we recently found that influenza C virus RDE can cleave O-acetyl
groups not only from Neu5,9Ac₂ but from several other O-acetylated sialic acids produced by chemical modification of Neu5Ac or Neu5Gl (F. Kitame et al., unpublished results), raising the possibility that the activity of influenza C virus O-acetylesterase may not be restricted to neuraminate-9-O-acetyl esters. Thus whether or not O-acetylated sialic acids other than Neu5,9Ac₂ can also be receptor determinants for attachment of influenza C virus remains to be determined.

It is known that influenza C virus agglutinates the erythrocytes from some species (mouse, rat, hamster and chicken) but not those from others (sheep, horse, ferret, pig, monkey and guinea-pig) (Minuse et al., 1954; Chakraverty, 1978; Rogers et al., 1986). The recent study of Rogers et al. (1986) suggested that the agglutination of vertebrate erythrocytes correlated well with the presence of Neu5,9Ac₂ on their surface. The ability of influenza C virus to agglutinate human erythrocytes, however, remains unexplained; although human erythrocytes from some individuals were agglutinated in high titres (Minuse et al., 1954; Chakraverty, 1978; Ohuchi et al., 1978), the presence of O-acetylated sialic acids, including Neu5,9Ac₂, has never been detected on the erythrocyte membrane (Corfield & Schauer, 1982).

In this report, we studied the interaction of influenza C virus with human erythrocytes from 35 different individuals. Binding experiments with radioactively labelled virus revealed that all of the specimens tested possessed influenza C virus-specific receptors susceptible to both neuraminidase and viral O-acetylesterase, suggesting that a low level of O-acetylated sialic acids, though not detected as yet, are present on the human erythrocyte membrane. Additionally, evidence is presented which suggests that the majority of the erythrocyte receptors reside on sialoglycoproteins, particularly on glycoporphin A.

METHODS

Virus and cells. The Ann Arbor/I/50 strain of influenza C virus was grown in the MDCK line of canine kidney cells in the presence or absence of 3 μCi/ml [3H]glucosamine (5 to 10 Ci/mmol; CEA, Gif-Sur-Yvette, France) and purified as described previously (Sugawara et al., 1981). The MDCK cells were grown in Eagle's MEM containing 10% bovine serum.

Preparation of erythrocytes. Erythrocytes were collected either by venipuncture (chicken, rat and human) or by heart puncture (mouse and guinea-pig) into 0.2 vol. of phosphate-buffered saline (PBS) pH 7.2 containing 4% (w/v) sodium citrate, washed three times in PBS and suspended in the same buffer. Cells were used within 2 days of preparation.

Haemagglutination titration. This was performed as described by Ohuchi et al. (1978) in microtitre plates, using a 0.5% suspension of chicken erythrocytes or a 1.0% suspension of human erythrocytes.

Binding of [3H]glucosamine-labelled virions to erythrocytes. The erythrocyte samples, suspended in PBS at the concentrations given in the text, were pelleted, washed twice in PBS and resuspended in 100 μl of the buffer containing 20000 c.p.m./ml of [3H]glucosamine-labelled influenza C virions with a specific activity of 150 c.p.m./haemagglutination unit (HAU). The mixture was then incubated at 0 °C for 1 h with frequent mixing. At the end of the incubation period, the cells were extensively washed with cold PBS, lysed in 300 μl of Protosol (New England Nuclear), and the amount of radioactivity associated with the cells was measured in 3 ml of scintillation fluid consisting of 96% (v/v) toluene and 4% (v/v) Liquifluor (New England Nuclear).

Determination of blood groups. Blood groups of human erythrocytes [ABO, MN, Lewis a and Lewis b, P1 and Rh (CcDEe)] were determined by haemagglutination using monoclonal antibodies against M, N, Lewis a and Lewis b (Biotest Diagnostics, F.R.G.) and polyclonal antibodies against A, B, Rh (CcDEe), and P1 antigens (Ortho Diagnostic Systems, N.J., U.S.A.).

Neuraminidase treatment of erythrocytes. To 1 ml of human erythrocyte suspension (10%, v/v) in BBS buffer (0-01 M-sodium diethylbarbiturate pH 7-0, 0-15 M-NaCl) containing 0-6% bovine serum albumin, 500 U/ml of penicillin and 500 μg/ml of streptomycin, 40 mU of Arthrobacter ureafaciens neuraminidase (Nakarai Chemicals, Kyoto, Japan) was added. The mixture was then allowed to react for 5 h at 37 °C. The erythrocytes were then washed three times in PBS and assayed for virus-binding capacity as described above.

Protease treatment of erythrocytes. A suspension of human erythrocytes (20%, v/v) in 50 μl PBS was incubated at 37 °C for 4 h in the presence or absence of ficin (Wako, Osaka, Japan), bromelain (Nakarai Chemicals), TPCK trypsin (Millipore), α-chymotrypsin (Miles Laboratories) or V-8 protease (Miles Laboratories). The enzymes were all used at 5 mg/ml. At the end of the incubation period, the erythrocytes were washed three times with PBS to remove the protease and then assayed for their receptor activity.
Influenza C virus receptor

Treatment of erythrocytes with influenza C virus. The washed erythrocytes (6 × 10^6 cells) were resuspended in 100 μl of PBS containing 12 HAU of purified virions and incubated at 37 °C for 5 h with frequent mixing. After being washed three times with PBS, cells were used for virus binding assays.

Treatment of erythrocytes with anti-M and anti-N monoclonal antibodies. Human erythrocytes (6 × 10^6 cells) were washed in PBS and resuspended in 50 μl of an undiluted solution containing monoclonal antibody (obtained from Biotest Diagnostics) reactive with either the M or N blood group antigen. After an incubation period of 15 min at room temperature, cells were washed three times in PBS and then tested for their virus-binding capacity.

Monoclonal antibodies against influenza C virus glycoprotein. Two monoclonal antibodies (J14 and S16) against the HE protein of influenza C virus, prepared and characterized as described previously (Hongo et al., 1986; Sugawara et al., 1986), were used after purification by 'salting out' with saturated ammonium sulphate. Our previous study showed that J14 inhibited haemagglutination activity, haemolytic activity and infectivity of the C/Ann Arbor/1/50 strain of influenza C virus whereas S16 did not inhibit any of them.

RESULTS

Agglutination of human erythrocytes by influenza C virus

The ability of influenza C virus to agglutinate human erythrocytes has not been reproducible (Minuse et al., 1954; Chakraverty, 1978; Ohuchi et al., 1978; Rogers et al., 1986). To examine this problem, human erythrocytes from 35 different individuals were tested for their ability to be agglutinated by the Ann Arbor/1/50 strain of influenza C virus. As has been described by Ohuchi et al. (1978), three different haemagglutination patterns were observed: 17 specimens showed clear agglutination with various HA titres, 13 exhibited partial agglutination even at the lowest dilution of virus tested, and five were negative. To investigate the possibility that the agglutination of human erythrocytes might be associated with the presence of a particular blood group antigen on their surface, representative blood groups including ABO, MN, Lewis, P1 and Rh were determined for 13 of the 35 specimens. The results, summarized in Table 1, indicate that the haemagglutination pattern of human erythrocytes is not a simple function of any of the blood groups tested. As described previously by Ohuchi et al. (1978), however, the preferential agglutination of type B erythrocytes was evident: a clear positive pattern was seen with six of seven type B erythrocytes but with only three of 18 type A erythrocytes.

Binding of radioactively labelled influenza C virus to human erythrocytes

To study the interaction of influenza C virus with human erythrocytes in more detail, we attempted to measure the binding of [3H]glucosamine-labelled C/Ann Arbor/1/50 virions to the cells. In Fig. 1, the ability of the labelled virus to bind to erythrocytes from four different species

Table 1. Comparison of the ability of influenza C virus to agglutinate human erythrocytes with different blood group antigens

<table>
<thead>
<tr>
<th>HA* pattern</th>
<th>Subject</th>
<th>ABO</th>
<th>M</th>
<th>N</th>
<th>Lewis a</th>
<th>Lewis b</th>
<th>P1</th>
<th>Rh</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>S.H.</td>
<td>A</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CeDEe</td>
</tr>
<tr>
<td></td>
<td>S.F.</td>
<td>B</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CeDEe</td>
</tr>
<tr>
<td></td>
<td>Y.M.</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ccDEE</td>
</tr>
<tr>
<td></td>
<td>K.I.</td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>CCDeE</td>
</tr>
<tr>
<td></td>
<td>M.H.</td>
<td>B</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CeDEe</td>
</tr>
<tr>
<td></td>
<td>K.T.</td>
<td>O</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>CCDeE</td>
</tr>
<tr>
<td></td>
<td>A.N.</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td>ND†</td>
<td>ND</td>
<td>-</td>
<td>CCDeE</td>
</tr>
<tr>
<td></td>
<td>F.N.</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td>ND†</td>
<td>ND</td>
<td>-</td>
<td>CCDeE</td>
</tr>
<tr>
<td>±</td>
<td>S.S.</td>
<td>A</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CeDEe</td>
</tr>
<tr>
<td></td>
<td>T.S.</td>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>ceDeE</td>
</tr>
<tr>
<td>-</td>
<td>M.K.</td>
<td>B</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>CCDeE</td>
</tr>
<tr>
<td></td>
<td>S.N.</td>
<td>O</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>CeDeE</td>
</tr>
<tr>
<td></td>
<td>H.N.</td>
<td>O</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>CCDeE</td>
</tr>
</tbody>
</table>
* +, Clearly agglutinated; ±, incompletely agglutinated; −, not agglutinated.  
† ND, Not determined.
Fig. 1. Attachment of influenza C virus to erythrocytes from different species. [3H]Glucosamine-labelled influenza C virions (2000 c.p.m. in 100 μl PBS) were incubated at 0 °C for 1 h with erythrocytes from mouse (●), rat (▲), chicken (○) and guinea-pig (△). After being washed extensively with PBS, cells were measured for radioactivity by scintillation counting. Each point is the mean of two determinations.

(mouse, rat, chicken and guinea-pig) was compared. The highest levels of adsorption were observed with mouse and rat erythrocytes, both of which contain a relatively high content of Neu5,9Ac₂ and are thus agglutinated by influenza C virus in high titres (Chakraverty, 1978; Shukla & Schauer, 1982; Rogers et al., 1986). A significant level of adsorption, though to a lesser extent than mouse and rat cells, was also seen with chicken erythrocytes. These cells are agglutinated well by influenza C virus, and contain Neu5,9Ac₂ (30 to 40% of total sialic acids) (Herder et al., 1987). In contrast to these cells, very few virions attached to the erythrocytes from guinea-pig which are known not to be agglutinated by influenza C virus (Minuse et al., 1954; Chakraverty, 1978). These observations support the validity of the present assay system.

Using this method, binding of influenza C virus to human erythrocytes from 35 different individuals was examined (Fig. 2). Significant binding, over the level of binding to guinea-pig erythrocytes, was detected with erythrocytes from almost all of the subjects. Although the level of virus attachment was markedly different from individual to individual, its correlation with haemagglutination pattern was evident. The proportion of bound virus, however, was as low as 5% of input even with the erythrocyte specimen (6 x 10⁶ cells) that showed the highest level of virus binding, the values being about 11 and three times lower than those obtained with the corresponding number of mouse or rat and chicken erythrocytes, respectively.

Specificity of influenza C virus binding to human erythrocytes

It was important to determine whether or not binding of influenza C virus to human erythrocytes is mediated through a specific recognition of cellular receptors by the HE protein of the virus. This was done using two anti-HE monoclonal antibodies (J14 and S16). Fig. 3 shows that binding of influenza C virus to the cells was blocked efficiently by J14 which has a strong haemagglutination inhibition activity but not by S16 which lacks this activity.

Recently, it has been reported that bacterial neuraminidases are able to destroy influenza C virus receptors on chicken erythrocytes as well as on tissue culture cells (Herrler et al., 1985a, b, c; Kitame et al., 1985; Herrler & Klenk, 1987). To see whether this is also the case with human erythrocytes, cells from two different persons were treated with neuraminidase from A. ureafaciens and then tested for influenza C virus binding. The results (Table 2) indicate that
Influenza C virus receptor

Fig. 2. Attachment of influenza C virus to human erythrocytes. Erythrocytes (6·0 × 10^6 cells) from 35 different individuals were suspended in 100 μl PBS containing 2000 c.p.m. of [3H]glucosamine-labelled influenza C virions and incubated at 0 °C for 1 h. At the end of the incubation period, unadsorbed virus was removed by washing in PBS, and cells were analysed for radioactivity. Each point indicates the mean of two replicates. The erythrocyte specimens tested were divided into three groups based on their haemagglutination patterns: -, not agglutinated; ±, incompletely agglutinated; +, clearly agglutinated. The dashed line indicates the level of virus binding to guinea-pig erythrocytes.

Fig. 3. Effect of anti-HE monoclonal antibodies on influenza C virus binding to human erythrocytes. [3H]Glucosamine-labelled influenza C virions (4000 c.p.m. in 100 μl PBS) were mixed with an equal volume of PBS containing various concentrations of monoclonal antibody J14 (●) or S16 (○), and were allowed to react at room temperature for 30 min. Untreated and antibody-treated virions were then measured for their ability to bind to 6·0 × 10^6 human erythrocytes according to the procedures described in Methods. Each point indicates the mean value of triplicate samples. The control sample had 802 ± 42 counts/10 min.

Table 2. Effect of treatment with neuraminidase or influenza C viral O-acetyesterase on virus attachment to chicken and human erythrocytes*

<table>
<thead>
<tr>
<th>Erythrocytes†</th>
<th>Neuraminidase</th>
<th>O-Acetyesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3294 ± 140‡</td>
<td>460 ± 18§</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K.T.</td>
<td>895 ± 34</td>
<td>229 ± 24§</td>
</tr>
<tr>
<td>S.H.</td>
<td>ND†</td>
<td>ND</td>
</tr>
<tr>
<td>H.N.</td>
<td>133 ± 19</td>
<td>60 ± 28§</td>
</tr>
</tbody>
</table>

* Binding experiments were done in three (chicken, K.T. and S.H.) or ten (H.N.) replicates, using 6·0 × 10^6 erythrocytes for each assay.
† Haemagglutination patterns of K.T. and S.H. were clearly positive, and that of H.N. was negative.
‡ Counts/10 min (mean ± S.D.).
§ Significance in the reduction of virus binding was confirmed by Student’s t-test (P < 0·005).
|| ND, Not determined.
neuraminidase treatment of human erythrocytes, under the conditions where the receptors on chicken erythrocytes were found to be destroyed almost completely, resulted in a drastic decrease in the number of bound virus particles. It should be noted that the effect of neuraminidase treatment on virus binding was observed with both of two specimens, one of which (H.N.) was negative in the haemagglutination test and displayed the lowest level of influenza C virus binding in the experiment shown in Fig. 2.

To examine the effect of influenza C virus esterase on virus attachment to human erythrocytes, cells from three different human samples, including H.N., were treated with influenza C virus at 37 °C for 5 h, and then virus binding assays were performed. As seen in Table 2, this treatment caused a marked reduction in the extent of virus binding to any of three erythrocyte specimens. To rule out the possibility that the virions used for pretreatment may have occupied the virus attachment sites thus blocking the binding of radioactively labelled virions, the following experiment was carried out with one erythrocyte specimen (S.H.). [3H]Glucosamine-labelled virions (12 HAU in 100 μl PBS) were added to 6.0 x 10⁶ erythrocytes and were adsorbed at 0 °C for 1 h. Either immediately after the adsorption period, or after a subsequent incubation at 37 °C for 5 h, cell-associated radioactivity was determined. The data indicated that the virions, which had attached to the cells at 0 °C, were mostly (approx. 92%) eluted during the following incubation at 37 °C (data not shown); this confirmed that the destruction of virus attachment sites by influenza C virus esterase was responsible for the reduction in the level of virus binding observed in Table 2.

These observations strongly suggest that the binding of influenza C virus to human erythrocytes occurs as a result of a specific interaction between viral HE glycoproteins and O-acetylated sialic acid-containing glycoconjugates on the cell membranes.

**Effect of proteolytic enzymes on influenza C virus binding to human erythrocytes**

To determine whether human erythrocyte receptors for influenza C virus are glycoproteins or glycolipids, cells were treated with various proteases (5 mg/ml for each) and measured for their receptor activity by binding assays. As can be seen in Table 3, virus attachment to human erythrocytes was inhibited almost completely by treatment with ficin, bromelain or V-8, suggesting that O-acetylated sialic acid-containing glycoproteins are the cellular receptors for influenza C virus. It was of interest to note in Table 3 that influenza C virus attachment to trypsin-treated cells and to α-chymotrypsin-treated cells was about 50% and 100% of that to untreated cells, respectively. These values were repeatedly obtained even when erythrocytes were treated with higher concentrations (15 mg/ml) of these enzymes.

**Table 3. Effect of protease treatment on influenza C virus attachment to human erythrocytes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Radioactivity bound†</th>
<th>Fraction of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>880 ± 32</td>
<td>100</td>
</tr>
<tr>
<td>Ficin</td>
<td>39 ± 1</td>
<td>4</td>
</tr>
<tr>
<td>Bromelain</td>
<td>49 ± 27</td>
<td>6</td>
</tr>
<tr>
<td>V-8</td>
<td>137 ± 20</td>
<td>16</td>
</tr>
<tr>
<td>Trypsin</td>
<td>444 ± 2</td>
<td>50</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>901 ± 26</td>
<td>102</td>
</tr>
</tbody>
</table>

* An erythrocyte specimen that showed a positive haemagglutination pattern was used. Binding assays were done in triplicate, using 6.0 x 10⁶ cells for each.
† Counts/10 min (mean ± s.d.).
Table 4. Influenza C virus binding to human erythrocytes treated with anti-M and anti-N antibodies*

<table>
<thead>
<tr>
<th>Subject</th>
<th>M and N antigens</th>
<th>Antibody</th>
<th>Radioactivity bound†</th>
<th>Fraction of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y.M.</td>
<td>M(+) N(+)</td>
<td>None</td>
<td>685</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M</td>
<td>179</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-N</td>
<td>219</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M + anti-N</td>
<td>77</td>
<td>11</td>
</tr>
<tr>
<td>K.T.</td>
<td>M(+) N(−)</td>
<td>None</td>
<td>822</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M</td>
<td>236</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-N</td>
<td>642</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M + anti-N</td>
<td>166</td>
<td>20</td>
</tr>
<tr>
<td>S.H.</td>
<td>M(−) N(+)</td>
<td>None</td>
<td>1024</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M</td>
<td>1116</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-N</td>
<td>157</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-M + anti-N</td>
<td>207</td>
<td>20</td>
</tr>
</tbody>
</table>

* Each binding assay was done using 6.0 × 10⁶ erythrocytes.
† Each value is the mean of two determinations (counts/10 min).

Adamany, 1978). To examine the possibility that glycophorin A may be involved in influenza C virus binding to these cells, virus attachment to three erythrocyte specimens, the M and N blood group activities of which were different from each other, was measured after treatment with either anti-M or anti-N monoclonal antibody. Table 4 shows that each of the antibodies greatly inhibited virus binding to the erythrocytes that had the corresponding antigen on their surface. The formation of small but visible aggregates was observed after treatment of human erythrocytes with anti-M or anti-N monoclonal antibody. The aggregates, however, could be readily dispersed by mixing on a thermomixer. Furthermore, treatment of erythrocytes with anti-Rh (D) polyclonal antibodies caused a similar degree of aggregate formation but did not inhibit virus binding significantly (data not shown). It appears, therefore, that the observed inhibition of virus binding by anti-M or anti-N monoclonal antibody is not due to the formation of erythrocyte aggregates, but is presumably due to the masking of virus-binding sites by the antibody molecules bound to glycophorin A.

**DISCUSSION**

The present study revealed that influenza C virus-specific receptors, known to be glycoconjugates containing O-acetylated sialic acid residues (Rogers et al., 1986; Herrler et al., 1987), are present on the surface of human erythrocytes though their number varied considerably among individuals. It is known that the major sialic acid in man is Neu5Ac. O-Acetylated sialic acids, particularly Neu5,9Ac₂, have also been shown to exist, though in low levels, in brain (Haverkamp et al., 1977), saliva (Haverkamp et al., 1976), colonic mucins (Rogers et al., 1978), B lymphocytes (Kamerling et al., 1982) and several melanoma cell lines (Cheresh et al., 1984). No biochemical evidence has been presented yet, however, for the presence of O-acetylated sialic acids in human erythrocytes (Corfield & Schauer, 1982). Identification of O-acetylated sialic acids on human erythrocyte membranes in this study suggests that the influenza C virus binding assay is much more sensitive in detecting these types of sialic acid than any of the chemical methods previously used.

Although the number of influenza C virus receptors on human erythrocytes remains to be determined, there are few compared with those on mouse and rat erythrocytes: less than 5% of added influenza C virions attached to human erythrocytes under conditions where 50 to 60% of the input bound to mouse or rat erythrocytes. As described previously (Minuse et al., 1954; Ohuchi et al., 1978) and in this report (see Table 1), the pattern as well as the titre of haemagglutination of human erythrocytes was variable among the individuals even when assayed at 4 °C. Rogers et al. (1986) also described that a transient agglutination only was
observed at room temperature even with erythrocytes that showed a stable agglutination pattern at 4 °C. These ambiguous results concerning the ability of influenza C virus to agglutinate human erythrocytes could be explained by the number of receptors present on the cells being insufficient to maintain a firm interaction with influenza C virus.

The results of Tables 3 and 4 strongly suggest that most of the influenza C virus-binding sites on human erythrocytes reside on the major glycoprotein, glycophorin A. It is still possible, however, that glycophorin B, which has a sequence identical to the N form of glycophorin A for at least the first 23 N-terminal amino acids (Furthmayr, 1978), may also be involved in influenza C virus binding. Treatment of M(+) N(−) erythrocytes with anti-N antibody inhibited virus attachment by about 20% while anti-M treatment of M(−) N(+) cells did not inhibit at all (see Table 4), raising the possibility that part of the receptor activity of human erythrocytes might be associated with glycophorin B. It is clear, however, that this species of glycoprotein is not the sole erythrocyte receptor for influenza C virus since trypsin treatment of human erythrocytes caused an approx. 50% reduction in virus binding (see Table 3). Glycophorin B has been shown to be resistant to cleavage by trypsin (Anstee, 1981).

The data shown in Table 3 allow us to speculate further on the possible site on glycophorin A recognized by influenza C virus. Treatment of human erythrocytes with chymotrypsin releases a peptide corresponding to amino acids 1 to 34 of glycophorin A (Tomita & Marchesi, 1975). Influenza C virus was found to attach to chymotrypsin-treated cells as efficiently as to untreated cells, suggesting that this region, which contains 11 O-linked and one N-linked oligosaccharide chains, is not essential for virus binding. Trypsin cleaves glycophorin A to release two peptides containing amino acids 1 to 31 and 32 to 39 (Tomita & Marchesi, 1975). The binding of influenza C virus to trypsin-treated cells was about 50% of that to untreated cells. It seems, therefore, that amino acids 35 to 39 and the O-linked oligosaccharide chain at position 37 (threonine) are partly responsible for influenza C virus attachment. The region spanning amino acids 40 to 70, which remains exposed on the erythrocyte after trypsin treatment and possesses three O-linked oligosaccharide chains at positions 44 (serine), 47 (threonine) and 50 (threonine), also appears to be important for virus binding. We suggest that O-acetylation of sialic acid may occur on at least one of three oligosaccharide chains at positions 44, 47 and 50 as well as on the oligosaccharide chain at position 37.

We wish to thank Drs Tsuneo Suzuki and Kazuo Umetsu (Department of Forensic Medicine, Yamagata University School of Medicine) for their helpful discussions. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a Grant-in-Aid of the Japan Medical Association.

REFERENCES


Influenza C virus receptor


(Received 6 April 1988)