Haemolysis by Two Alphaviruses: Semliki Forest and Sindbis Virus

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SUMMARY

Purified preparations of Semliki Forest (SFV) and Sindbis virus haemolysed red blood cells from several species of animals and birds. The optimal haemolysis by SFV was obtained at pH 5.8 with 1-day-old chick erythrocytes incubated at room temperature. Considerable variation in haemolytic activity was observed between different virus preparations purified by different methods. The haemolytic activity of SFV was inhibited by antisera against whole virus or isolated envelope proteins but not with antiserum against virus capsid protein. Neither lipid and detergent-free envelope protein octamers with high haemagglutinating titre, nor isolated nucleocapsids caused haemolysis. Fresh, unpurified SFV and Sindbis virus preparations did not haemolysed unless they were exposed for repeated cycles of freezing and thawing. It appears that the haemolytic activity resides in the virus glycoproteins(s) but can only be manifested in slightly damaged whole virus particles.

INTRODUCTION

Haemolysis was first described for mumps virus (Morgan et al. 1948) followed by Newcastle disease virus (Kilham, 1949). Other paramyxoviruses, Sendai virus (Fukai & Suzuki, 1955), measles virus (DeMeio, 1962) and SV5 virus (Scheid et al. 1972; Scheid & Choppin, 1973), also have haemolytic activity which is a property of the smaller surface glycoprotein, F-protein, which is also responsible for the cell fusing activity of these viruses (Hall & Martin, 1974; Scheid & Choppin, 1974; Seto et al. 1974). Lipid free paramyxovirus envelope protein preparations do not cause haemolysis though they retain haemagglutinating activity. The haemolytic activity can be restored by addition of lipids to the protein preparations (Hosaka & Shimizu, 1972; Neurath et al. 1973; Hall & Martin, 1974).

The haemolytic activity of Sendai virus can be demonstrated over a wide pH range (pH 5 to 8) and has a temperature optimum of 37°C (Clavell & Bratt, 1972). Fresh, unfrozen Sendai virus preparations ('early harvest') do not exhibit haemolytic activity but they can cause cell fusion (Homma et al. 1976).

Crude togavirus preparations from infected mouse brain and tissue culture fluids have also been reported to have haemolytic activity (Karabatsos, 1963, 1965).

Here we show that two purified alphaviruses, Sindbis and SFV, can haemolysed several different kinds of red blood cells. The haemolytic activity is a property of virus glycoprotein(s) but can only be demonstrated in virus particles probably damaged during the purification process.
METHODS

Viruses. Semliki Forest virus (SFV), prototype strain, was cultivated in BHK21 cells in Eagle’s minimal essential medium (MEM) containing 0.2% bovine serum albumin (Kääriäinen et al. 1969). After removal of cell debris by centrifugation at 10000 g for 30 min, the supernatant fluid was concentrated either by vacuum dialysis (Kääriäinen et al. 1969) or by precipitation with polyethylene glycol (Davis, 1971).

The concentrated virus was purified by isopycnic banding in a discontinuous sucrose gradient made in TN buffer (0.14 M-NaCl, 0.01 M-Tris, pH 7.4; Söderlund et al. 1972). The virus band was diluted with this buffer and virus particles were sedimented at 175000 g for 1.5 h in a Spinco SW50.1 rotor. The pellet was resuspended in TN buffer at a protein concentration of 5 mg/ml and stored at −70°C in small samples.

Purified Sindbis virus, HR strain (Burge & Pfefferkorn, 1966), was obtained through the courtesy of Dr Carl-Henrik von Bonsdorff of this department. The virus had been concentrated with polyethylene glycol and purified by sucrose density gradient centrifugation (Strauss et al. 1969). The virus was stored at −70°C in TN buffer containing 0.001 M-EDTA. The protein concentration was 2.4 mg/ml.

Fresh SFV and Sindbis virus were prepared by infecting BHK21 cells with 50 p.f.u./cell. After an adsorption period of 60 min the inoculum was removed, cells were washed twice with growth medium and the cultures incubated at 37°C for 7 to 10 h in MEM supplemented with 0.2% bovine serum albumin. The cell debris was removed at 10000 g for 30 min and the supernatant virus was used directly in assays for haemolytic and haemagglutinating activity. After dilution with an equal vol. of borate buffered saline, pH 9, supplemented with 0.2% bovine serum albumin (Clarke & Casals, 1958), a portion of the virus at pH 9 was pelleted before analysis in an SW27 rotor for 2.5 h at 25000 rev/min.

Isolation of nucleocapsid and envelope protein fractions. 35S-methionine-labelled Semliki Forest virus, purified as described previously (Kääriäinen & Söderlund, 1971), was mixed with 1 mg of purified unlabelled SFV in 1 ml of TN buffer containing 1% Triton X-100. The nucleocapsids were separated in a 15 to 30 (w/w) sucrose gradient made in TN buffer. After centrifugation for 2 h in a SW41 rotor at 39000 rev/min at 4°C, fractions of 0.3 ml were collected by sucking from the bottom of the tube with a peristaltic pump. Trichloroacetic acid-insoluble radioactivity was determined in a sample of each fraction. The nucleocapsid fractions were pooled and used immediately for determination of haemolytic and haemagglutinating activity.

To separate the lipid- and detergent-free envelope protein octamers sedimenting at 29S the envelope proteins at the top of the gradient were subjected to further centrifugation in 20 to 50% (w/w) sucrose gradients in TN buffer for 21 h at 39000 rev/min in an SW41 rotor at 20°C (Helenius & von Bonsdorff 1976).

Immune sera. Rabbits were immunized with purified SFV, envelope protein octamers (29S) and capsid protein isolated nucleocapsids by phenol extraction (Rueckert & Schäfer, 1965). Calcium phosphate and complete Freund’s adjuvant were used as adjuvants for envelope and capsid proteins respectively. The rabbits were injected 3 to 5 times at 1 to 2 week intervals and bled 2 weeks after the last injection.

Haemagglutination and haemagglutination inhibition tests. The haemagglutination assays were carried out on microtitration plates at pH 5.8, using 50 μl of virus dilution in borate saline, pH 9 and 50 μl of 1% suspension of red blood cells in phosphate buffer, giving a final pH of 5.8 (Clarke & Casals, 1958).

For haemagglutination inhibition, the sera were treated with acid-washed kaolin at pH 9 to remove non-specific inhibitors (Clarke & Casals, 1958), and were diluted 1 to 10 during this process. About 8 haemagglutinating units (HAU) of the antigen (polyethylene glycglycol-
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precipitated SFV) were added to serial dilutions of serum made in borate-saline. After overnight incubation at 4°C, 1% red blood cells were added to the wells and the test was read after 1 h incubation at room temperature.

**Haemolysis and haemolysis inhibition test.** Virus dilutions were made in borate-saline, pH 9, supplemented with 0.2% bovine serum albumin. To 0.5 ml of a virus dilution, 0.5 ml of 1% red blood cells in phosphate buffer was added. The tubes were incubated for 30 min at room temperature in the standard assay, mixing the contents thoroughly every 10 min. At the end of the incubation period the red blood cells were sedimented at 500 g for 10 min. The absorbance of the supernatant fluid was measured in a Zeiss PMQ II spectrophotometer at 540 nm. The results were expressed as per cent haemolysis (%HL) compared with the maximal 100% obtained by sonication of the corresponding erythrocyte suspension. One haemolytic unit (HLU) was defined as the dilution causing 20% haemolysis.

For the haemolysis inhibition test the sera were treated with acid-washed kaolin at pH 9 (Clarke & Casals, 1958), diluting the sera initially 1:10. From each serum dilution made in borate-saline buffer containing 0.2% bovine serum albumin, 250 µl was mixed with 250 µl of virus dilution corresponding to 20 HLU/ml. After overnight incubation at 4°C, 0.5 ml of a 1% suspension of day-old chicken erythrocytes in phosphate buffer was added to each tube. Incubation was at room temperature for 30 min. The degree of haemolysis was read as described. The results were expressed at %HL from the maximal haemolysis obtained without serum. The per cent inhibition (%HLI) is thus 100% minus percentage HL obtained.

**Red blood cells.** Red blood cells from man (type O), goose, rooster, guinea pig, 1-day-old chicken and African green monkey, were obtained from Orion Diagnostica, Helsinki, Finland. The cells were washed immediately three times with dextrose-gelatin veronal (Clarke & Casals, 1958) and preserved as 10% suspension in the same solution. The red blood cell concentration was determined in an automatic cell counter, Coulter Counter Model Industrial D.

**Complement-fixation test.** The antibody titres of sera from rabbits immunized with purified capsid protein were determined in complement fixation test. Nucleocapsids isolated by sucrose gradient centrifugation were used as antigens in a box titration carried out in microtitration plates as described previously (Klemola & Kääriäinen, 1965).

**Protein** was determined according to Lowry et al. (1951).

**Isolation of IgG fraction.** Immunoglobulin fractions from the rabbit antisera were isolated according to Palacios et al. (1972).

**RESULTS**

**Optimal conditions for haemolysis**

SFV concentrated by vacuum dialysis and purified by isopycnic banding in sucrose gradients was tested for haemolysing activity using red blood cells from several different species. Virus dilutions were made in borate saline, pH 9, containing 0.2% bovine serum albumin. Red blood cells were added in phosphate buffer to give a final concentration of 0.5% and pH 5.8. Haemagglutination titrations were made from the same virus dilutions on microtitration plates. After 30 min incubation the red cells in the haemolysis test were sedimented by low speed centrifugation and the absorption from the supernatant fluids was measured at 540 nm. The degree of haemolysis was expressed as percentage of maximal haemolysis obtained by sonication. The dilutions corresponding to 50% and 20% haemolysis were estimated from curves similar to those shown in Fig. 3. The haemagglutination and haemolysis titres measured with several kinds of red blood cells are shown in Table 1. As reported previously, the goose red blood cells are most sensitive in the haemagglutination
Table 1. Comparison of haemolysis and haemagglutination by SFV with erythrocytes from different species

<table>
<thead>
<tr>
<th>Erythrocyte</th>
<th>HAU/ml</th>
<th>HLU/ml (20 % HL)</th>
<th>HLU/ml (50 % HL)</th>
<th>HAU/μg</th>
<th>HLU/μg (20 % HL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goose</td>
<td>144 000</td>
<td>34 000</td>
<td>8 400</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>1-day-old chicken</td>
<td>72 000</td>
<td>48 000</td>
<td>131 000</td>
<td>14</td>
<td>96</td>
</tr>
<tr>
<td>Rooster</td>
<td>64 000</td>
<td>32 000</td>
<td>100 000</td>
<td>13</td>
<td>64</td>
</tr>
<tr>
<td>African green monkey</td>
<td>64 000</td>
<td>188 000</td>
<td>58 000</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>Human type O</td>
<td>51 200</td>
<td>64 000</td>
<td>20 000</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>&lt;1 600</td>
<td>&lt;1 600</td>
<td>&lt;4 000</td>
<td>5</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Sheep</td>
<td>&lt;1 600</td>
<td>&lt;1 600</td>
<td>&lt;4 000</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of the incubation temperature on the haemolytic activity of SFV: 125 ng of SFV was incubated in a 0.5 % suspension of 1-day-old chicken erythrocyte cells for 30 min.

Fig. 2. pH optimum of the haemolysis by Semliki Forest virus: 60 ng of SFV was incubated in 0.5 % suspension of 1-day-old chicken erythrocytes for 30 min.

test of alphabiruses (Clarke & Casals, 1958). However, several other types of erythrocyte also gave high titres. The best haemolysis was obtained with erythrocytes from 1-day-old chicken irrespective of whether 50 % or 20 % haemolysis was taken as the end point of titration. Since the 20 % haemolysis can be measured reliably it was taken as the end-point, i.e. the virus dilution causing a 20 % haemolysis was regarded as containing one haemolytic unit per ml. By this criterion haemolysis was a more sensitive titration method than haemagglutination, except with guinea pig erythrocytes which showed haemagglutination but no haemolysis. Both haemagglutination and haemolysis titres are also expressed per μg of virus protein in Table 1.

The pH and temperature optima were determined using the same SFV preparation and chicken red blood cells. The optimum incubation temperature was between 20 and 30°C (Fig. 1) and the pH optimum was 5.8. No haemolytic activity could be demonstrated at pH 6.6 or higher (Fig. 2). In this respect SFV and Sindbis were unlike the paramyxoviruses.

The correlation between haemagglutination titre, protein concentration and percentage of haemolysis was determined for Sindbis virus, SFV concentrated with polyethylene glycol and the SVF preparation used in the previous experiments. Fig. 3 shows that for the three virus preparations there is a linear correlation between virus concentration and HA titre. On the other hand, the correlation between percentage haemolysis and virus concentration...
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Fig. 3. Correlation between haemagglutination titre and protein concentration (○—○, SFV-a; □—□, SFV-b; △—△, SIN), as well as between % haemolysis and protein concentration (●•●, SFV-a; ■•■, SFV-b; ▲•▲, SIN) measured using 0.5% 1-day-old chicken erythrocytes. Sindbis virus and SFV-b were concentrated by precipitation with polyethylene glycol. SFV-a was concentrated by vacuum dialysis and purified by banding in sucrose gradients including several centrifugations to pellet the virus. Horizontal bars represent the standard deviation of the haemolysis measurements at 20% and 50% haemolysis.

gives slightly sigmoid curves which have an almost linear section between 20% and 80% haemolysis. The shape of the curves closely resembles the theoretical curve presented by Bratt & Clavell (1972) based on an assumption that in principle one particle can cause haemolysis of one red blood cell. The Sindbis (SIN) virus preparation clearly had less haemolytic activity than the two SFV preparations. Interestingly, the SFV preparations concentrated by different methods also showed considerable differences in haemolytic but not haemagglutinating activities. Assays with a given virus preparation were highly reproducible as demonstrated by the small standard deviations at 20% and 50% haemolysis points (Fig. 3).

Inhibition of haemolysis by immune sera

Sera from normal and immunized rabbits were tested for their ability to inhibit haemolysis caused by SFV. Untreated normal rabbit sera had variable inhibitory activity which could be removed by kaolin treatment or isolation of the IgG fraction from the serum. Immune serum against purified virus particles had moderately high titre in both haemagglutination and haemolysis inhibition tests, when these were carried out with the same amount of virus
Table 2. Inhibition of haemolysis and haemagglutination with 8 HAU SFV-b by antisera (or IgG) against whole virus, envelope proteins and nucleocapsids*

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>50% HLI</th>
<th>20% HLI</th>
<th>HI-titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole virus, serum</td>
<td>1024</td>
<td>2048</td>
<td>640</td>
</tr>
<tr>
<td>Whole virus, IgG</td>
<td>1280</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>Envelope serum</td>
<td>200</td>
<td>400</td>
<td>80</td>
</tr>
<tr>
<td>Envelope, IgG</td>
<td>512</td>
<td>1024</td>
<td>320</td>
</tr>
<tr>
<td>Capsid, serum</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Normal rabbit serum before kaolin treatment</td>
<td>64</td>
<td>256</td>
<td>10</td>
</tr>
<tr>
<td>Normal rabbit serum after kaolin treatment</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Normal rabbit IgG</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>

* Sera were treated with kaolin to remove non-specific inhibitors. Anticapsid serum had an antibody titre of 32, measured by complement fixation.

Table 3. The effect of repeated freezing and thawing on haemolytic and haemagglutinating activity of SFV and Sindbis virus*

<table>
<thead>
<tr>
<th>Virus material</th>
<th>Cycles of freezing and thawing</th>
<th>HAU/ml</th>
<th>HLU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFV culture medium</td>
<td>None</td>
<td>128</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>128</td>
<td>48</td>
</tr>
<tr>
<td>SFV pellet</td>
<td>None</td>
<td>1024</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>512</td>
<td>144</td>
</tr>
<tr>
<td>Sindbis culture medium</td>
<td>None</td>
<td>64</td>
<td>&lt; 1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>Sindbis pellet</td>
<td>None</td>
<td>512</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>512</td>
<td>10</td>
</tr>
</tbody>
</table>

* Viruses were harvested at 10 h p.c. The titres were measured of cell culture fluids and of pelleted and resuspended virus preparations.

(Table 2). Under these conditions two- to fourfold higher titres were obtained for the sera by haemolysis inhibition compared with haemagglutination inhibition, depending on whether the 50% or 20% inhibition of haemolysis was taken as the end-point.

Antiserum against purified envelope proteins inhibited both tests whereas antiserum against capsid protein had no inhibitory effect in either test.

Nature of the haemolysis

Inhibition of the haemolysis by antiserum against isolated envelope proteins suggests that these glycoproteins are responsible for the phenomenon. To investigate this we isolated envelope protein octamers, sedimenting at 29S, and the nucleocapsid fractions of the SFV as described in Methods and tested their haemagglutinating and haemolyzing activities. As expected, nucleocapsids were inactive in both tests. The 29S fraction had a high haemagglutination titre (20480 HAU/ml), but showed no haemolytic activity (< 1 HLU/ml). Similar results have been reported for paramyxoviruses (Hosaka & Shimizu, 1972a; Hall & Martin, 1974) and also for crude Eastern equine encephalitis virus preparations (Karabatsos, 1973).

Finally, fresh preparations of SFV and Sindbis virus were tested for their haemolyzing activity. No haemolysis could be demonstrated despite the presence of considerable amounts of haemagglutinin (Table 3). Freezing and thawing ten times made SFV clearly haemolytic but affected Sindbis virus only slightly. Pelleting of SFV made it haemolytic whereas Sindbis virus showed haemolysis only after five cycles of freezing and thawing. However, in all cases
the HA/HL ratio was higher than in purified virus preparations. Incubation of the culture medium for several weeks in borate saline pH 9 at 4°C did not release haemolysing activity from either of the viruses.

**DISCUSSION**

The original finding of haemolytic activity in several togaviruses reported by Karabatsos (1963, 1965, 1973) has largely been neglected (Pfefferkorn & Shapiro, 1974; Strauss & Strauss, 1976; Kääriäinen & Söderlund, 1978), possibly because of uncertainty of the virus specificity of the phenomenon. We have confirmed the findings by Karabatsos (1963, 1965, 1973) by showing that purified Sindbis virus and SFV can haemolysse red blood cells from several different mammals and birds. The haemolytic activity of SFV was high enough for antibody assays to be performed using purified virus as the antigen. By this means it was possible to show that the virus glycoproteins are responsible for the haemolysis caused by these viruses.

The isolated envelope protein octamers were, however, unable to cause haemolysis even when used at 20000 times the quantity of purified virus. It appears that whole virus particles are needed for haemolysis to occur. These findings are compatible with results obtained for different paramyxoviruses. The surface glycoproteins $F_1 + F_2$ (Gething et al. 1978; Scheid & Choppin, 1977) are responsible for the haemolysis, for the cell fusion activity and infectivity of these viruses (Scheid & Choppin, 1974; Seto et al. 1974). If the proteolytic cleavage of $F_0$, the precursor for $F_1$ and $F_2$, is inhibited for some reason, non-infectious, haemagglutinating virus particles without haemolysing or cell fusion activity are released into the medium (Homma, 1972; Homma & Ohuchi, 1973; Scheid & Choppin, 1974, 1976).

We have shown that newly released unfrozen SFV and Sindbis virus are incapable of haemolysis although they agglutinate red blood cells. Sedimentation in an ultracentrifuge and several cycles of freezing and thawing made the Semliki Forest viruses haemolytic, supporting the findings of Karabatsos (1965) and Shimizu et al. (1976).

It is apparent that during the purification process the virus particles become slightly damaged as is also reflected in their 70 to 90% loss of infectivity (Kääriäinen et al. 1969). Interestingly, Sindbis virus seemed to be more stable to physical stresses than SFV.

Despite the ‘artefactual’ nature of the haemolysis phenomenon of paramyxoviruses, it reflects an essential property of the virus, namely the ability to initiate infection (Scheid & Choppin, 1974). The same is probably true for alphaviruses.

Our attempts to identify the protein responsible for the haemolysis by using antisera against denatured envelope proteins $E_1$ and $E_2$ have failed. By analogy with the paramyxoviruses, $E_2$ would be a probable candidate for the haemolysis activity. Like $F$ proteins, $E_2$ is cleaved from a larger precursor, $p62$ ($pE_2$) (Schlesinger et al. 1972; Simons et al. 1973). In the case of SFV the other cleavage product, $E_3$, remains at the surface of the virus in close association with $E_2$ and $E_1$ (Ziemiecki & Garoff, 1978) again in analogy with the paramyxoviruses, where $F_1$ and $F_2$ remain together after the proteolytic cleavage has taken place (Gething et al. 1978).

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