Role of Viral Glycoproteins in Haemolysis by Influenza B Virus

(Accepted 9 November 1981)

SUMMARY

Influenza B virus exhibited haemolytic activity at low pH, but the pH profile for its activity varied from strain to strain. The results of selective heat-inactivation of the enzyme activity of neuraminidase (NA) or elimination from virions of the enzyme-active portion of the NA molecule by trypsin digestion, suggest that proteolytically cleaved haemagglutinin, but not enzyme activity of NA, is essential for haemolysis by influenza B virus.

Both the haemagglutinin (HA) of myxoviruses and the F glycoprotein of paramyxoviruses are involved in the initiation of infection and are activated by proteolytic cleavage (Lazarowitz & Choppin, 1975; Klenk et al., 1975; Homma & Ohuchi, 1973; Scheid & Choppin, 1974; Nagai & Klenk, 1977). The HA of myxoviruses is cleaved to yield disulphate-bonded HA₁ and HA₂ polypeptides (Klenk et al., 1975; Lazarowitz & Choppin, 1975). The initial N-terminal amino acid sequence of the HA₂ polypeptide resembles that of the F₁ polypeptide of paramyxoviruses, responsible for cell fusion and haemolysis. Synthetic oligopeptides containing the initial N-terminal sequence of these polypeptides competitively inhibit infection by these viruses (Skehel & Waterfield, 1975; Ward & Dopheide, 1979; Waterfield et al., 1979; Richardson et al., 1980; Choppin et al., 1981). From the structural and functional similarities between HA and F, a possible fusion activity of myxovirus HA was suggested, although there was no definite evidence for the involvement of the fusion reaction in the process of penetration of myxoviruses.

Recently, it has been found that liposomes containing both cleaved HA and neuraminidase (NA) of influenza A virus fuse with the cell membrane (Huang et al., 1980a, b), and that influenza A virions in cleaved form cause haemolysis and fusion by interacting with the cells at low pH (Huang et al., 1981; Lenard & Miller, 1981). Huang and co-workers (1980b, 1981) have indicated that, in addition to cleaved HA, the function of NA is required for fusion, based on evidence that liposomes with only cleaved HA express fusion activity in the presence of soluble NA of bacterial or viral origin, and that anti-NA antibody inhibits not only fusion by liposomes but also virus-induced fusion at low pH.

The function of myxovirus NA in replication has been extensively studied and the NA is thought not to be essential during the early steps of replication, but during the budding process (Bucher & Palese, 1975). Thus, it is important to investigate whether the NA of influenza B virus plays an essential role in fusion as well. Haemolysis by virus requires a single fusion event between the virus envelope and the cell membrane of erythrocytes (Bachi et al., 1977). In the present work we have studied the role of the NA of influenza B virus in haemolysis of chick erythrocytes at low pH.

The Lee/40, Kagoshima/68, Kanagawa/73, Hong Kong/73 and Amagusa/78 strains of influenza B virus were grown in the allantoic cavity of 10-day-old embryonated chicken eggs. The harvested allantoic fluids were clarified at 2000 rev/min for 15 min and used for haemolysis tests. A 3.5 ml vol. of allantoic fluid containing 0.5% chick erythrocytes in test tubes was kept at 4 °C for 30 min with occasional mixing. The erythrocytes were then pelleted, washed with chilled saline, and resuspended in the original volume of saline buffered with 0.1 M-sodium acetate to pH 5 or 5.4, or with 0.1 M-sodium phosphate to pH 5.9, 6.4 or
Short communications

Table 1. pH dependence of haemolysis by influenza B virus strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>HA†</th>
<th>7.2</th>
<th>6.4</th>
<th>5.9</th>
<th>5.4</th>
<th>5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Lee/40</td>
<td>128</td>
<td>0.008</td>
<td>0.009</td>
<td>0.020</td>
<td>0.020</td>
<td>0.116</td>
</tr>
<tr>
<td>B/Kagoshima/68</td>
<td>64</td>
<td>0.019</td>
<td>0.052</td>
<td>0.170</td>
<td>0.130</td>
<td>0.197</td>
</tr>
<tr>
<td>B/Kanagawa/73</td>
<td>256</td>
<td>0.002</td>
<td>0.028</td>
<td>0.510</td>
<td>0.365</td>
<td>0.370</td>
</tr>
<tr>
<td>B/Hong Kong/73</td>
<td>64</td>
<td>0.009</td>
<td>0.022</td>
<td>0.200</td>
<td>0.202</td>
<td>0.122</td>
</tr>
<tr>
<td>B/Amagusa/78</td>
<td>128</td>
<td>0.009</td>
<td>0.022</td>
<td>0.170</td>
<td>0.112</td>
<td>0.172</td>
</tr>
</tbody>
</table>

* All virus strains were grown in allantoic cavity of 10-day-old embryonated chicken eggs and the harvested allantoic fluids were used for the haemolysis test.
† Haemagglutinating activity per 0.25 ml of allantoic fluid.
‡ Haemolytic activity per 3.5 ml of allantoic fluid containing 0.5% chick erythrocytes.

Table 2. Effect of loss of neuraminidase activity of B/Kanagawa on viral haemolytic activity

(a) Alteration in viral activities of B/Kanagawa by heating at 55 °C*

<table>
<thead>
<tr>
<th>Heating time (min)</th>
<th>HA activity per 0.25 ml</th>
<th>Enzyme activity† (A_{540} per 0.1 ml)</th>
<th>Haemolytic activity‡ (A_{540} per 3.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>256</td>
<td>0.850</td>
<td>0.300</td>
</tr>
<tr>
<td>10</td>
<td>256</td>
<td>0.020</td>
<td>0.365</td>
</tr>
<tr>
<td>20</td>
<td>256</td>
<td>0.020</td>
<td>0.325</td>
</tr>
<tr>
<td>30</td>
<td>256</td>
<td>0.023</td>
<td>0.382</td>
</tr>
</tbody>
</table>

(b) Virion-associated and soluble biological activities after centrifugation of trypsin-treated B/Kanagawa‡

<table>
<thead>
<tr>
<th>Trypsin concentrate (μg/ml)</th>
<th>HA activity per 0.25 ml</th>
<th>Enzyme activity† (A_{540} per 0.1 ml)</th>
<th>Haemolytic activity‡ (A_{540} per 3.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>256</td>
<td>0.960</td>
<td>0.475</td>
</tr>
<tr>
<td>20</td>
<td>256</td>
<td>0.256</td>
<td>1.000</td>
</tr>
<tr>
<td>50</td>
<td>256</td>
<td>0.070</td>
<td>1.360</td>
</tr>
<tr>
<td>100</td>
<td>256</td>
<td>0.022</td>
<td>1.320</td>
</tr>
<tr>
<td>250</td>
<td>256</td>
<td>0.009</td>
<td>1.060</td>
</tr>
</tbody>
</table>

* Egg-grown B/Kanagawa suspended in allantoic fluid was used for this experiment.
† Enzyme activity was assayed with a fetuin substrate by a modification of Warren’s thiobarbituric acid method as described previously (Maeno & Kilbourne, 1970).
‡ Egg-grown B/Kanagawa, partially purified by centrifugation through a sucrose gradient, was used in this study.

7.2. After 30 min incubation in a water bath at 36 °C, the tubes were centrifuged at 1000 rev/min for 5 min and the released haemoglobin was determined from the A_{540} of the supernatant. As shown in Table 1, all virus strains tested were found to cause haemolysis at low pH, but these pH profiles differed among strains. B/Kanagawa had maximal haemolytic activity at pH 5.9 and B/Lee exhibited haemolytic activity at the lowest pH tested (pH 5) with negligible haemolysis above pH 5.4. The remaining strains showed appreciable haemolytic activity at pHs ranging from 5 to 5.9. Within these pH ranges there was no pH-dependent haemolysis in the absence of virus. A similar study was carried out with B/Kanagawa grown in chick embryo fibroblast cells. In contrast to egg-grown virus possessing cleaved HA, this virus did not cause haemolysis even at the optimal pH of 5.9, but by treatment with 10 μg/ml trypsin (Sigma) for 10 min it exhibited significant haemolysis (data not shown), suggesting that proteolytic cleavage of the HA is a prerequisite for virus-induced haemolysis. It must be noted that haemolysis by influenza B viruses at pH 5.9 accompanied the aggregation of chick erythrocytes and the virus did not elute from erythrocytes at all.
Experiments were undertaken to investigate the role of the enzyme activity of NA in haemolysis by influenza B virus. Egg-grown strain B/Kanagawa virus in allantoic fluid was kept in a water bath at 55 °C for various periods of time and assayed for the residual viral activities. More than 97% of the virus neuraminidase activity (A₄₉₀) was heat-inactivated within 10 min without any loss of HA activity (Table 2a). This was further confirmed by testing the capacity of these viruses to elute from chick erythrocytes. Agglutinated erythrocytes in saline were incubated at 36 °C for 30 min and after centrifugation the supernatants were assayed for HA activity. With all of the heated preparations, HA activity was not detected in the supernatant, in contrast to the control preparation where 100% of the original HA activity was recovered (data not shown). Each of the virus preparations (3-5 ml) was tested for haemolytic activity at pH 5.9 as described above. It was found that all of the heated preparations exhibited higher haemolytic activity than the control preparation.

The possible independence of haemolysis by B/Kanagawa on the enzyme activity of NA was confirmed using virions from which NA was removed by tryptic digestion. Egg-grown B/Kanagawa virus was partially purified by sedimentation on a cushion of 60% sucrose through 10% sucrose in a Spinco SW27 at 20000 rev/min for 1 h. The virus was suspended in phosphate-buffered saline (PBS, pH 7.2) and treated with various concentrations of crystal trypsin (Sigma) at 36 °C for 30 min. After the addition of soybean trypsin inhibitor (250 μg/ml, Sigma), the virus suspensions were centrifuged in a Spinco SW50 at 25 000 rev/min for 1 h. The pellets were resuspended in the original volume of PBS (pH 7.2) and, together with the supernatants, tested for HA and enzyme activity (Table 2b). It was found that at trypsin concentrations of 100 and 250 μg/ml, more than 98% of the virion-associated enzyme activity was released from the virions, but almost all HA was recovered in the sediment. The recovery of total neuraminidase enzyme activity consistently exceeded the input after treatment with trypsin. Each of the virus suspensions (3.5 ml) was tested for haemolytic activity at pH 5.9. Even the removal of more than 99% of the virus neuraminidase activity by exposure to trypsin (250 μg/ml) did not reduce viral haemolytic activity but rather induced higher haemolytic activity (Table 2b). Treated virus suspensions did not cause haemolysis when tested at pH 7.2.

Influenza A viruses and alphaviruses have been found to exhibit haemolytic activity when they interact with erythrocytes at a suitable pH (Maeda & Ohnishi, 1980; Huang et al., 1981; Lenard & Miller, 1981). The present study shows that influenza B viruses have the ability to induce haemolysis at low pH, and for this activity cleaved HA is required as with influenza A viruses. The role of the enzyme activity of the NA in haemolysis by influenza B virus was studied by heat inactivation or removal from virions by trypsin digestion of the virus enzyme activity. These treatments of influenza B virus, which caused the loss of almost all the virus enzyme activity, did not result in the decrease of viral haemolytic activity but rather induced higher haemolytic activity. These findings suggest that influenza B virus-induced haemolysis does not depend on the enzyme activity of the NA. We do not know why digestion of virions with trypsin and heat inactivation of enzyme activity of NA both enhanced viral haemolytic activity. It is evident that cleaved HA is essential for haemolysis by influenza B virus. Therefore, a possible explanation for this may be that the removal of the enzyme-active portion of the NA by trypsin digestion or a conformational change of the NA molecule following heat denaturation contributes to the interaction of HA₂ and the cell membrane of erythrocytes.

We thank Eriko Shibata and Fumi Yamamoto for their excellent technical assistance. This work was supported by research grants from the Ministry of Education, Science and Culture of Japan, and the Ministry of Health and Welfare, Japan.
Short communications

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(Received 4 September 1981)