Conditions for Haemolysis by Flaviviruses and Characterization of the Haemolysin

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(Accepted 15 July 1985)

SUMMARY

The 17D vaccine strain of yellow fever virus (YF 17D) was used to establish the optimal conditions for lysis of chick erythrocytes. Tissue culture-grown, polyethylene glycol-concentrated virus showed peak activity at pH 5.4 in citrate buffer when incubated at 37 °C. A further two- to fourfold increase in titre was obtained by pretreatment of the chick erythrocytes with 250 µg/ml trypsin. These conditions were also shown to be optimal for Japanese encephalitis (JE), West Nile (WN) and dengue-2 (den2) viruses. The ratio of haemagglutination (HA) titre to haemolysis (HL) titre approximated to unity, suggesting that the two functions are associated with the same molecule although as separable entities since selective inactivation of the HL activity of the virus was accomplished using 60 µg/ml trypsin. HL could be demonstrated at neutral pH if the chick erythrocytes were first subjected to treatment with acidic pH buffer. The effect on the virus envelope is thus not the sole contribution of a low pH environment to optimal HL. Hyperimmune rabbit antiserum prepared against purified YF 17D virions inhibited HA and HL if added before agglutination had occurred by the virus but when added after agglutination had taken place it showed specific anti-HL activity. Monoclonal antibodies that inhibited HA (HAI) by YF 17D did not inhibit HL (HLI) activity when applied after agglutination had taken place. Moreover, monoclonal antibodies specific for the 54K glycoprotein of YF virus but without HAI activity also had no effect on HL when added either before or after agglutination. As yet, we have been unable to identify a monoclonal antibody displaying specific anti-HL activity but all those directed against the 54K envelope glycoprotein possessing HAI activity showed HA to be a prerequisite for HL.

A number of viruses have been shown to lyse red blood cells and this property has been unequivocally associated with the viral envelope (Karabatsos, 1963; Howe & Lee, 1972, Väännänen & Kääriäinen, 1979; Huang et al., 1981). In the genus Paramyxovirus lysis results from fusion of an envelope glycoprotein with the erythrocyte membrane after initial contact with viral haemagglutinin (Homma & Ohuchi, 1973; Scheid & Choppin, 1974). In contrast, the orthomyxoviruses and alphaviruses appear to implement lysis directly using haemagglutinin (Skehel et al., 1982; Webster et al., 1983; Chanas et al., 1982), a property which is evident for alphaviruses only in slightly damaged whole virus particles (Väännänen & Kääriäinen, 1979). In the past, low levels of haemolytic (HL) activity have been reported for Japanese encephalitis (JE) virus at the pH which mediates maximal haemagglutinating (HA) activity (Karabatsos, 1965). However, the author failed to increase HL yields of several flaviviruses using methods successfully applied to alphaviruses. Evaluation of the optimal conditions for HL by paramyxoviruses, orthomyxoviruses and alphaviruses has demonstrated an acidic pH dependency which differs between the genera (Lenard & Miller, 1981) and indeed also between individual strains of influenza virus (Huang et al., 1981). Moreover, lysosomotropic amines, known to increase lysosomal pH in macrophages (Ohkuma & Poole, 1978), significantly inhibit...
infection of P388D1 macrophage-like cells by the flavivirus West Nile (WN) (Gollins & Porterfield, 1984) and have been shown more recently to inhibit infection of BHK-21 cells by frog virus 3 (Braunwald et al., 1985), suggesting that an acidic endosomal or lysosomal stage might be associated with virus entry. For flaviviruses the complexity of interactions between virus and cellular membranes and their significance in viral pathogenesis has yet to be fully explained. Characterization of biological functions attributable to the surface proteins of flaviviruses will contribute to our understanding of their role in viral pathogenesis. In this paper, we define optimal conditions for HL induced by flaviviruses and describe a procedure for selectively inactivating the HL activity.

Vero cells were subcultured in Leibovitz L15 medium containing 10% foetal calf serum (FCS), 10% tryptose phosphate broth, 100 units/ml penicillin and 100 μg/ml streptomycin (growth medium). Confluent monolayers were infected with either the vaccine strain of yellow fever virus (YF 17D; Wellcome Arilvax Batch No. BYF 1228), JE, WN or dengue-2 (den2) virus at an input multiplicity of 0:1 in medium containing 2% FCS (maintenance medium). The infected cells were incubated at 37 °C until at least 70% were rounded and detaching from the surface. The supernatant medium was clarified by centrifugation at 2000 g for 15 min. Virus was concentrated using 7% polyethylene glycol (PEG) and 0.4 M-NaCl at 4 °C overnight. The precipitate was recovered at 10 000g for 30 min and dissolved in borate-buffered saline at pH 9 (Clarke & Casals, 1958), producing a concentration factor of 100. Aliquots stored at −70 °C were thawed once only and typically contained 5 to 10 mg/ml protein. Twenty per cent infected suckling mouse brain suspensions of each flavivirus were also concentrated as above. HA and HA inhibition (HAI) tests followed the procedure of Clarke & Casals (1958) except that chick erythrocytes were used. Tests for HL activity were carried out initially at pH 6.2, which was the optimal condition for HA, by modification of the method of Maeda et al. (1981). One-hundred units of HA were mixed with an equal volume of 1% erythrocytes for 30 min at room temperature. Mixtures were then centrifuged at 2000 g for 5 min. The supernatant buffer was removed and replaced by 200 μl citrate buffer (0.85% NaCl, 0.1 M-citric acid, 0.1 M-Na2HPO4) adjusted to the appropriate pH. The mixtures were incubated at 37 °C for 2 h with gentle shaking at 10 min intervals. After centrifugation, HL was estimated by absorbance at 410 nm. For trypsin treatment, trypsin (Gibco 2.5%) was diluted in phosphate-buffered saline at pH 7.4 and mixed with equal volumes of either erythrocytes or virus for 1 h at 37 °C. The reaction was terminated for erythrocytes by washing in dextrose/gelatine–veronal buffer (Clarke & Casals, 1958), whereas for virus the mixtures were placed in an ice-bath. For acid treatment, chick erythrocytes were suspended in citrate buffer pH 5.4 for 5 min at room temperature, before washing in excess 0.85% saline pH 7.2 (adjusted with 10−3 M-NaOH; see Huang et al., 1985). Hyperimmune rabbit anti-YF virus (RH1) antiserum was prepared using purified YF 17D virus as described by Gould et al. (1985). This antiserum had a titre of 1:5120 when tested by HAI against YF 17D virus. Monoclonal antibodies prepared against YF and JE viruses and known to be specific for the 54K envelope glycoprotein of the appropriate virus have been described elsewhere (Gould et al., 1985).

A series of experiments was then performed to determine the optimal conditions for virus-specific lysis of chick erythrocytes. Initially, PEG-concentrated tissue culture and suckling mouse brain suspensions of either YF, JE or WN virus with HA titres in excess of 1:2560 were used in an attempt to lyse either gander or chick erythrocytes at pH 6.2. Conventional HA titrations (Clarke & Casals, 1958) were carried out. The microtitre plates were then shaken to resuspend the erythrocytes and the plates were incubated at 37 °C for up to 24 h. No lysis was observed with any of the virus preparations. The tests were then performed using citrate buffer for the second stage as described above. Each virus sample was adjusted to 100 HA units (HAU) and after incubation with the chick erythrocytes for 30 min the pelleted erythrocytes were resuspended in citrate buffer at pH intervals of 0.2 ranging from pH 5.0 to 7.0. Fig 1 shows the relative HL observed for tissue culture-derived YF 17D virus. Maximal lysis was recorded at pH 5.4. Similar results were obtained with tissue culture-derived JE, WN and den2 viruses. In our experience little or no HL activity was observed when infected suckling mouse brain material was used; however, the HA titres of these preparations were relatively low. No HL activity was
recorded using either uninfected PEG-precipitated tissue culture fluid or uninfected mouse brain antigen. Flavivirus HL occurred over a range of temperatures from 4 °C to 37 °C, performing optimally at the latter.

HL tests were then carried out at pH 5.4 but this time the viruses were titrated to determine the HA : HL ratio. In Fig. 2 it can be seen that the maximum lysis occurred with 50 or more HAU. In repeated tests the HA : HL ratio was 1 : 1 and this ratio was maintained even after 10 successive cycles of rapid freezing and thawing using liquid nitrogen. It is noteworthy that the freezing cycles had no significant effect on titres. However, a two- to fourfold increase in both the HA and HL titres was achieved by pretreatment of the erythrocytes with 250 μg/ml trypsin as described earlier, whereas in contrast, treatment of virus with 60 μg/ml trypsin for 1 h at 37 °C reduced the HL titre to less than 20% without affecting the HA titre (Fig. 3). Increasing the concentration of trypsin beyond 60 μg/ml led to the inactivation of both HA and HL. Treatment of the chick erythrocytes with citrate buffer at pH 5.4 before use in the HL test showed that HL still reached the level of controls with a standard preparation of chick erythrocytes.
Table 1. **Haemagglutination inhibition (HAI) and haemolytic inhibition (HLI) activities of a rabbit hyperimmune antiserum and monoclonal antibodies prepared using YF 17D virus**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pattern of precipitation</th>
<th>HAI*</th>
<th>Before agglutination</th>
<th>After agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1</td>
<td>gp54</td>
<td>5120</td>
<td>5120</td>
<td>320</td>
</tr>
<tr>
<td>864</td>
<td>gp54</td>
<td>20480</td>
<td>20480</td>
<td>-</td>
</tr>
<tr>
<td>813</td>
<td>gp54</td>
<td>5120</td>
<td>5120</td>
<td>-</td>
</tr>
<tr>
<td>825</td>
<td>gp54</td>
<td>160</td>
<td>160</td>
<td>-</td>
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<tr>
<td>612</td>
<td>gp54</td>
<td>320</td>
<td>320</td>
<td>-</td>
</tr>
<tr>
<td>427</td>
<td>gp54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>810</td>
<td>gp54</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>871</td>
<td>gp48</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Reciprocal of dilution at which HA was inhibited by 50%.
† Reciprocal of dilution at which HL was inhibited by 50%.

HL could also be recorded when, after agglutination of acid-treated cells, they were resuspended in phosphate buffer pH 6.2 (optimal for agglutination) or in 0.85% saline pH 7.2. In these instances, HL activity was 50% or less of that resulting from virus-induced lysis at pH 5.4. It is also interesting to note that lysis of a standard preparation of chick erythrocytes could be achieved if, after agglutination, the virus–cell complex was incubated at pH 6.2 or pH 7.2 following a period of 2 min in citrate buffer pH 5.4.

The ability of YF virus to lyse chick erythrocytes was shown to be dependent upon and secondary to HA using the RH1 antiserum and monoclonal antibodies specific for the 54K envelope glycoprotein (Table 1). In the first set of experiments, the antibodies were added to the virus preparations before the erythrocytes. Any antibodies that had previously displayed HAI activity inhibited agglutination and consequently no lysis occurred at pH 5.4. Antibodies that did not possess HAI activity did not inhibit HL. The experiments were repeated but this time the antibodies were added after agglutination had been allowed to take place. None of the monoclonal antibodies had any effect on lysis; however, the RH1 antiserum inhibited HL but its titre was at least tenfold lower than seen in HAI tests.

From the results described above it appears likely that HL will be a general property of flaviviruses. Many of the requirements for HL activity at acidic pH resemble those described for influenza viruses (Huang et al., 1981) which perhaps mediate lysis through the action of a hydrophobic amino-terminus region located near the base of the haemagglutinin molecule (Skehel et al., 1982; Webster et al., 1983). The amino acid sequence of this region of the polypeptide is reported to be highly conserved between strains and similar to the fusion peptide of the paramyxoviruses (Skehel & Waterfield, 1975). Our results suggest that the flaviviruses may mediate lysis by a mechanism similar to that suggested for influenza viruses since lytic activity in the citrate buffer system was associated specifically with the 54K envelope glycoprotein and no fusion protein analogues to that of the paramyxoviruses (Scheid & Choppin, 1973, 1974) has been identified in the flaviviruses. Virus-specific HL could not be demonstrated in the phosphate buffer system used for alphaviruses (Väänänen & Kääriäinen, 1979). Selective inactivation of HL by trypsin may therefore reflect a specific destruction of the active site or a non-specific alteration of the physical state of the haemagglutinin molecule. Huang et al. (1985) have demonstrated that HL by fowl plague virus could occur at neutral pH if the chick erythrocytes alone were treated with low pH buffer. Our results tend to support these findings, although HL activity only reached approximately 50% or less of maximal levels. We have confirmed, however, that the acidic pH has an immediate effect and the continued presence of the low pH buffer is not necessary to facilitate HL. As yet, we have been unable to identify monoclonal antibodies that can specifically inhibit flavivirus HL activity when added after agglutination has occurred. Hyperimmune antiserum, however, specific for the envelope
glycoprotein of YF virus, did show inhibition of HL under these conditions. This mechanism of HL inhibition is not yet known but it seems possible that steric hindrance occurs between the erythrocyte and virion when antibody molecules attach to the active HL site of the 54K polypeptide. Thus, in common with many other enveloped viruses HL is secondary to but dependent upon HA.

In the present study, we have described an HL activity for flaviviruses comparable to that previously demonstrated for orthomyxoviruses and alphaviruses. It is well known that the fusion (F) glycoprotein of paramyxoviruses is directly involved in virus penetration and cell-to-cell spread by fusion of virus and cell membranes (Homma & Ohuchi, 1973; Scheid & Choppin, 1974) and that anti-F antibodies are able to block these functions (Merz et al., 1980, 1981). Although anti-HL antibody does not neutralize infectivity of Sindbis virus (Chanas et al., 1982) and non-HL strains of Semliki Forest virus can exist which cause cell fusion (Väänänen & Kääriäinen, 1980), this activity is still an indicator of the fusogenic activity of the virus envelope proteins (Väänänen et al., 1981). Whether or not our observations are pertinent to understanding the pathogenesis of flaviviruses remains to be seen but it is hoped that analysis of plaque variants will help clarify the role of HL in virus virulence. This work is currently in progress.

We are very grateful to Miss S. Faulder for typing the manuscript. This work was supported by a grant from the Medical Research Council. Grant No. G8219412/T.

REFERENCES


Short communication


(Received 11 March 1985)