Hemolytic Activity of a Togavirus, Getah*

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Abstract A purified toga-alphavirus, Getah (GET), showed optimal hemolytic activity for one-day-old chick red blood cells when incubated at 37°C for 120 min at pH 6.2. Experimental data obtained from various angles, such as pH dependency, inhibition by virus-specific antiserum and by concanavalin A, indicated that the hemolysis was a property of the virus particle itself. Although the mechanism of hemolysis by togaviruses has not been known, our results indicated that viral lipids may participate in this activity since the hemolytic activity was impaired by delipidation procedures.

A number of enveloped, RNA-containing viruses have hemolytic (HE) activity. Among these are paramyxoviruses such as Sendai (HVJ), mumps, measles, and Newcastle virus (NDV) (2, 6, 7, 11) and togaviruses (8-10). Since togaviruses such as Sindbis virus are comparatively simple in their chemical nature, they provide a model for studying the mechanism of the activity. This report describes studies on the HE activity of a representative togavirus, Getah (GET). Conditions which remove the HE activity but not the hemagglutinating activity are discussed, and the HE reactions of purified GET virus are compared to those previously investigated using crude virus extracts (8-10).

MATERIALS AND METHODS

Virus and cells. GET virus, AMM2021 strain (kindly supplied by Dr. Oya, the National Institute of Health, Tokyo, and passed through suckling mice intracerebrally for 2 to 4 generations in our laboratory) was cultivated in BHK-21 cells for one to three passages to eliminate defective interfering virus (14). The method of cultivation of BHK-21 cells and viruses were the same as previously described (15).

Purification of virus. The virus was purified as described before (16). The virus in infected culture fluid was precipitated by zinc acetate (0.05 M) and resuspended in saturated EDTA solution (pH 7.8). After the suspension was centrifuged at 100,000 g for 60 min at 4°C, the pellet was resuspended in STE buffer (0.13 M NaCl, 0.01 M Tris-hydrochloride and 10^-3 M EDTA, pH 7.2), which was

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then subjected to sucrose density gradient (10 to 40% (wt/vol)) centrifugation at 100,000 g for 50 min at 4°C. The virus collected as a visible band was diluted with STE and centrifuged at 100,000 g for 60 min at 4°C. The final suspension of the pellet was made in a small volume of STE, which was then diluted with buffered solution for each experiment. Figure 1 shows the final virus preparation used in this experiment; apparently no cellular materials are noted.

**Hemagglutination (HA) and hemagglutination-inhibition (HI) of virus.** HA and HI titers were determined according to the method of Clarke and Casals (3).

**Hemolysis (HE) and hemolysis-inhibition (HEI) of virus.** These tests were performed in the same buffer system as that for the HA tests of arboviruses (3). To the virus suspension (usually 0.4 ml) which had been diluted with borate-buffer saline (pH 9.0, BS9) containing 0.2% bovine serum albumin (Fraction V, Sigma Chemicals), was added an equal volume of various concentrations of one-day-old chick red blood cells (CRBC) in virus adjusting diluents (VAD; usually the assays were done at pH 6.2). After an incubation at 37°C for 120 min with occasional shaking, the mixture was centrifuged at 1,000 g for 10 min at room temperature; the supernatant was diluted to 4.0 ml with phosphate-buffer saline from which Ca++ and Mg++ were omitted (PBS(-), pH 7.0 (5)) and the optical density at 530 nm was determined. Two control blanks, one containing only CRBC and another containing CRBC plus 0.1% Nonidet P40 (NP40) in the same buffer solution, were used as 0% and 100% lysis standards. HE activity was expressed as percentage of 100% lysis standard or values of the optical density at 530 nm.

For HEI assays, 0.2 ml of virus (16 HAU/ml) diluted with BS9 was added with 0.2 ml of serial dilutions of acetone treated-antiserum and concanavalin A (Con A) in BS9; after 60 min at 37°C, a 1% CRBC suspension was added and HE activity were determined as described above.

**Ether treatment of virus.** Precooled anhydrous diethyl ether (ten volumes) was mixed with one volume of the virus suspension. The mixture was vigorously shaken.
for 10 min in an ice bath, then the supernatant ether was removed, fresh ether added and the treatment was repeated for an additional 10 min. Residual ether was removed by bubbling with nitrogen gas.

Acetone and acetone-ether treatment. Precooled acetone (ten volumes) was mixed with one volume of the virus solution. The mixture was shaken vigorously, the precipitate was collected by centrifugation at 1,000 g for 10 min at 4°C, and the treatment was repeated. The precipitate was dried in vacuo, dissolved into BS9, and then a portion thereof was treated with ether as mentioned above to obtain acetone-ether treated virus materials.

NP40 treatment of virus. To NP40 serially diluted with PBS (—) was added an equal volume of a virus suspension; after 10 min at 4°C, aliquots of the mixtures were taken for determination of HE and HA titers.

Pancreatin treatment of virus. One gram of pancreatin (Wako Chemicals, Osaka, crude powder of bovine pancreas) was suspended in STE, vigorously shaken for 30 min at room temperature, centrifuged at 3,000 g for 10 min at room temperature and then the supernatant was passed through a membrane filter (Millipore, 0.45 μm). The filtrate was serially diluted with STE (0.5 ml), added to the virus suspension in STE (0.5 ml) and incubated at 37°C for 10 min. Then portions thereof were used for HE and HA assays and another (0.5 ml) for analysis on a sucrose density gradient.

Fig. 2. Degree of hemolysis produced by increasing incubation times at various dilutions. Mixtures of serially diluted virus in BS9 and 0.5% CRBC in VAD (final pH 6.2) were incubated at 37°C for the indicated times, and then HE activities were determined at 530 nm, as described in the text. Symbols: 30 min (○), 60 min (●), 90 min (△), 120 min (▲) and 180 min (□).
Sucrose density gradient centrifugation. Two-tenths to 0.3 ml of the acetone or pancreatin treated virus samples were laid onto 4.5 ml of a linear 10 to 40% (wt/vol) or 10 to 30% (wt/vol) gradient of sucrose in STE and centrifuged at 38,000 rpm for appropriate hours at 4 C in a RPS65T rotor of a Hitachi 65P centrifuge. Fractions for analysis were collected through a hole punctured at the bottom of the tube. Each fraction was diluted ten-fold with BS9 in an ice bath; 0.4 ml of diluted samples mixed with 0.4 ml of 2% CRBC in VAD was used for HE determination, and the remainder for HA assay.

Electron microscopy. The virus in STE was stained with 2% uranyl acetate (pH 4) and 0.75% phosphotangstic acid (pH 7) on a carbon-coated grid. The specimens were photographed by an electron microscope (Japan Electron Co., Model JE 100B) at a magnification of ×50,000.

Immune serum. Anti-GET antiserum was prepared from a male rabbit by the foot-pad or subcutaneous administration of 2 ml of a mixture of 1 mg purified virus (in 1 ml) and 1 ml of Freund's complete adjuvant. Two doses were given at an interval of 2 weeks and the serum was obtained 10 days after the second inoculation.
HEMOLYTIC ACTIVITY OF TOGAVIRUS

Table 1. Effects of antiserum and Con A on the hemolytic activity of GET virus

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>Antiserum dilution</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>10^6(a)</td>
<td>0.000(c)</td>
<td>0.067</td>
</tr>
<tr>
<td>10^-1</td>
<td>0.000</td>
<td>0.045</td>
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<tr>
<td>10^-2</td>
<td>0.000</td>
<td>0.015</td>
</tr>
<tr>
<td>10^-3</td>
<td>0.000</td>
<td>0.012</td>
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<tr>
<td>Con A (µg/ml)</td>
<td>1,000</td>
<td>250</td>
</tr>
<tr>
<td>10^6(b)</td>
<td>0.148(c)</td>
<td>0.222</td>
</tr>
</tbody>
</table>

(a) 16 HAU/ml.
(b) 640 HAU/ml.
(c) O.D. at 530 nm.

RESULTS

Hemolytic Activity of GET Virus

The hemolytic (HE) activity of purified GET virus is demonstrated in Fig. 2. Serial dilutions of virus in BS9 and 0.5% CRBC in VAD were incubated at 37°C; the hemolysis proceeded gradually and the maximum hemolysis took place after 120 min. The degrees of hemolysis appeared to be dependent on the virus concentration when the virus was not in excess.

The pH dependency of togavirus HA is such a well-known criterion that it has sometimes been used to determine whether or not a newly isolated virus belongs to the togavirus group. Hemolytic activity is also pH dependent. As shown in Fig. 3, the most intensive HE activity was observed at pH 6.2 and pH 6.0. The HA activity of the same samples at pH 6.0, pH 6.2, pH 6.4, pH 6.6, pH 6.8 and pH 7.0 were 512, 512, 16, 2, <2 and <2 (per 0.4 ml), respectively. These results indicate that the HE activity is virus-specific, and that there is a relationship between the HA and HE activities. However, as discussed later, there are some viral components which possess only the HA activity.

When the virus suspensions were treated with the antiserum or Con A which reduces HA activity of virus (16), the HE activity was inhibited, as shown in Table 1. A low dilution of antiserum completely inhibited the HE activity of the virus, while high concentrations of Con A inhibited 50% of the HE activity.

Other Properties of the Hemolytic Activity

The HE reaction occurred over a wide temperature range as described by Karabatsos (8). Although it proceeded at room temperature as well as at 4°C, a longer time was required for complete hemolysis at 4°C.

Hemolysis was inhibited by CaCl₂; a concentration of 1.25 mM CaCl₂ inhibited 45% of hemolysis. One mM, 0.75 mM and 0.5 mM of CaCl₂ inhibited 38%, 25% and 15% of the HE activity, respectively.

The HE activity was not destroyed by ultraviolet irradiation; infectivity
of GET virus was lost (below 0.0001%) within a few min, but 95% of the HA and HE activity remained after 10 min of irradiation. Thus, a non-nucleic acid component must be the major ingredient of the HE reactivity.

**Effects of Solvents and NP40 on the Hemolytic Activity**

To demonstrate whether or not the HA and HE activities are caused by the same viral components, the virus was treated by various procedures. Acetone, acetone-ether and ether treatments of the virus produced roughly the same effects on the HA and HE activities. No differences were demonstrated between the untreated and treated samples, except for a slight increase of the HA activity (data not shown). However, sucrose density gradient analysis of the acetone-treated sample showed some slowly sedimenting materials with HA activity but no HE activity (Fig. 4). Most of the HA activity sedimented to the bottom and exhibited HE activity; this may be consistent with the fact that acetone treatment could extract only 10% of phospholipids from the virus samples (data not shown). This indicates that HA components lacking the HE activity can be separated from the component bearing both the HA and HE activities.

Delipidation of virus leads to loss of the HE activity; treatment by NP40 demonstrates this clearly. Figure 5 shows the effect of NP40 on the HE and HA activities of the virus. The HE activity was reduced greatly by all the NP40 concentrations above 0.25%. The HA activity was not reduced by the NP40 treatment; titers (HAU/0.4 ml) of 625, 3,125, 3,125 and 3,125 were obtained after treatment with 0, 0.25, 0.5 and 1% of NP40 concentrations, respectively. Triton X-100 treatment of virus caused complete dissociation of envelope glycoproteins from viral envelopes and the resulting glycoproteins had HA activity (13). These findings indicate that envelope lipids components play an important role in the HE activity.
Pancreatin Treatment of Virus

From the results of NP40 treatment of virus, the HE activity seems to require intactness of viral envelope components, especially of lipid components. Another experiment demonstrating this was done as follows; virus was treated with pancreatin solution as described in Methods, and then subjected to sucrose density gradient centrifugation. Treatment of virus with a 10-fold dilution of pancreatin reduced the HE activity by 60%; the HA activity increased 2 to 4 times. The pancreatin solution alone had no effect on hemolysis of CRBC. As shown in Fig. 6 (A), (B), the pancreatin treated virus sedimented more slowly and more heterogeneously (B) than untreated intact virus (A). The logarithm of HE/HA ratio(×10³) of the untreated virus is above zero (A), and the same values are obtained at around fraction 10 of the treated sample (B) which represent the sedimentating position of intact virus. However, the main peak of the pancreatin-treated virus (B) has lower HE/HA ratios(×10³) (approaching −1) than those of the untreated virus (A). This indicated that the differential loss of the HE activity was produced presumably by delipidation activity of pancreatic lipase contained in crude pancreatin. These support a hypothesis that virus lipids play an essential role in hemolysis.

DISCUSSION

The hemolytic activity of togavirus has been reported previously by Karabatsos (8–10), but the fundamental mechanism of hemolysis of chick red blood cells has not yet been studied.

In most of the reported experiments on viral HE, the HA and HE activities
have been found to be closely associated (2, 6, 7, 11). However, in experiments using various treatments presently performed which resulted in separation of HA and HE components, we have demonstrated that the HE activity of Getah virus required both HA and lipid components; HA with no trace of HE activity can occur and furthermore, partial or complete removal of lipid results in a partial destruction of the HE activity, leaving the HA activity relatively intact. It seems that envelope glycoprotein E4 (which has been demonstrated to have hemagglutinin activity by Darlymple et al (4)) first binds to chick red cell membrane lipid

Fig. 6. Sucrose density gradient centrifugation of pancreatin treated virus. Untreated virus sample (A) and pancreatin treated virus sample (B) (3 times more concentrated than the untreated) were centrifuged on 10 to 40% (wt/vol) sucrose density gradients at 38,000 rpm for 50 min at 4°C, and HE(○) and HA(●) activities were determined. Dotted line represents the values in log (HE/HA × 10^3).
components (phospholipids or cholesterol, or both (12)), and then viral lipid components may interact with cellular membrane lipids. If one assumes that virus membranes fuse with the cell membrane (1), there might be many possible chances for viral components, both proteins and lipids, to influence the response of the CRBC membrane eventually leading to lysis. It may be that removal of lipid from the particle prevents the fusion step and consequently prevents hemolysis; this may be the case of partial delipidation with acetone-ether treatment and NP40 treatment of the virus. Which viral membrane lipid components (phospholipids, cholesterol or others) are responsible for the HE activity is not clear at the present moment. To distinguish the responsible factor, it will be useful to test virus particles with altered lipid composition (produced by growth in different hosts or further delipidation by specific phospholipases) for their HE activity.

REFERENCES