Haemagglutination-inhibition Test for Haemorrhagic Fever with Renal Syndrome Using Virus Antigen Prepared from Infected Tissue Culture Fluid

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SUMMARY

Haemagglutinating (HA) antigens of four strains of virus related to that causing haemorrhagic fever with renal syndrome (HFRS) were prepared from infected tissue culture fluids by ultracentrifugation. The titres of the precipitated antigens were increased considerably by acetone extraction and sonication. Acetone extraction completely inactivated infectious virus in the antigen preparations. The antigens were pH-dependent, with pH optima at 5.8. Good correlations were observed in human and rat sera between the titres obtained by the haemagglutination-inhibition (HI) test and an indirect fluorescent antibody test. Moreover, strong cross-reactions among these strains were demonstrated by the HI test. The HI test has not been used previously with HFRS viruses because of the danger involved in preparing HA antigen, but these results indicate that a safe method is available for serological identification of HFRS.

INTRODUCTION

Haemorrhagic fever with renal syndrome (HFRS) is a severe and widespread infectious disease. Many epidemics of HFRS in China, Korea and the far-eastern region of the Soviet Union have been reported (Lee, 1982). In Japan, more than 100 researchers using experimental rats have contracted HFRS in the last 10 years. The aetiological agent of the disease was first isolated by Lee et al. (1978) and subsequently many virus strains that are antigenically related to HFRS have been isolated in various parts of the world. We also isolated an HFRS-related virus, named strain B-1, from a tumour taken from a Fischer rat (Yamanishi et al., 1983).

Serological diagnosis of HFRS has been carried out mainly by the indirect immunofluorescent (IF) test, which is a simple test that does not involve any pretreatment of test sera. However, it does require a fluorescence microscope and species-specific conjugates. Moreover, reliable results can be obtained only when samples are examined by an experienced observer, and the subjective nature of the test has sometimes resulted in difficulty in comparing serological data obtained in different laboratories. Therefore, a haemagglutination inhibition (HI) test, which is now the most popular and standardized serological test for viral diseases, is desirable for detection of HFRS.

Development of an HI test has been hampered by the difficulty of preparing HFRS virus haemagglutination (HA) antigen, because the virus is classified as one of the dangerous pathogens and can be handled only in class P3 laboratories. Tsai et al. (1984) first succeeded in preparing HA antigen of HFRS (Hantaan) virus from infected suckling mouse brain (SMB). However, homogenization of virus-infected SMB is dangerous, resulting in particulate spread of the virus in the air. To avoid this difficulty, we prepared HA antigen from infected culture fluid and here we report preparation of antigen of high titre and its use in an HI test.
METHODS

Viruses. The HFRS-related virus strains used were B-1 (Yamanishi et al., 1983), Hantaan virus (HV) strain 76-118 (Lee et al., 1978), Prospect Hill (PH) virus (Lee et al., 1982) and Hfillnfis (Hs) virus (Yanagihara et al., 1984). They were passaged in Vero E6 cells in our laboratory.

Cell culture. Vero E6 cells were obtained from the American Type Culture Collection and grown in medium containing a mixture of medium 199 and Eagle’s MEM supplemented with 10% foetal calf serum (FCS) for growth medium and 3% FCS for maintenance medium.

Preparation of HFRS-related virus HA antigens. Vero E6 cells were infected with HFRS-related viruses at an m.o.i. of about 0.01 TCID₅₀/cell and cultured for 1 to 2 weeks. The infected culture fluids were used as starting materials for preparation of HA antigens. Fig. 1 shows the procedures used for HA antigen (B-1 strain) preparation. One-hundred ml of infected fluid (F) was centrifuged at 7000 r.p.m. for 30 min to precipitate the cell debris (P-1), and the supernatant (S-1) was re-centrifuged at 25000 r.p.m. at 4 °C for 2 h in a Beckman 30 rotor. The supernatant (S-2) was discarded and the precipitate (P-2) was suspended in 2 ml bovine albumin–borate–saline solution (BABS pH 9) and extracted twice with chilled acetone (A) and then once with ether (AE) and dried. The resulting residues of the respective extracts were reconstituted with borate–saline (BS pH 9). The material extracted with acetone was further treated by sonication for 3 min (AS-3’) or 10 min (AS-10’). These samples (P-2, A, AE, AS-3’, AS-10’) could be used as HA antigen.

HA test. The HA test was performed by the method of Shope & Sather (1979) using goose erythrocytes (RBC) and 96-well plastic plates (Termo, Tokyo, Japan). The antigen dilutions were tested at different pH values of 5.67 to 7.0 and the optimum pH for each virus was determined. The antigens were incubated with RBC at 37 °C for 30 min, and the endpoint of the HA reaction (1 unit) was taken as the highest dilution at which complete HA occurred.

HI test. The HI test was performed by the method of Shope & Sather (1979). Test sera were extracted twice with chilled acetone and dried. The residues were resuspended in BS pH 9, at 1:10 dilution based on the volume of infected tissue culture fluid: 100 ml

\[
\text{Infected tissue culture fluid: 100 ml} \\
(F) \\
\text{Centrifugation 7000 r.p.m. 30 min} \\
\text{Precipitate} \\
\text{Supernatant} \\
\text{Suspend in 2 ml BABS pH 9 (S-1)} \\
\text{Centrifugation 25000 r.p.m. 2 h} \\
\text{Precipitate} \\
\text{Supernatant} \\
\text{Suspend in 2 ml BABS pH 9 (S-2)} \\
\text{Acetone extraction} \\
\text{Acetone–ether extraction (A) (AE)} \\
\text{Sonication 3 min} \\
\text{Sonication 10 min (AS-3') (AS-10')} \\
\text{Fig. 1. Protocol for HA antigen (B-1 strain) preparation.}
\]
HI test for HFRS virus

serum initially introduced, and left to stand overnight at 4 °C. On the following day, they were adsorbed with packed RBC. The HA test was performed before the HI test and 8 HA units at the optimum pH (5.8) were used in the HI test. Serially diluted test sera in a microplate were mixed with an equal volume (25 μl/well) of HA antigen and incubated at 37 °C for 1 h. Then the mixtures were added with 0.33°~ RBC (50 μl/well) and inhibition of the HA pattern was read after incubation at 37 °C for 30 min. If the pattern was not clear, the plates were left at room temperature for several hours more.

Indirect IF test. Antibody titres in test sera were measured by the indirect IF test described by Yamanouchi et al. (1984). Briefly, Vero E6 cells infected with the HFRS virus, B-1 strain, were trypsinized and suspended in growth medium. Aliquots of the cell suspension were seeded on glass slides, fixed with acetone and stained by the indirect IF test with human or rat sera as the first antibodies. The second antibodies used were fluorescein isothiocyanate-conjugated anti-human or rat IgG rabbit sera (Cappel Laboratories, Cochranville, Pa., U.S.A.).

Infectivity assay. The infectivity of samples of strain B-I was assayed by the method of Tanishita et al. (1984) involving focus counting. Infectivity was expressed in focus-forming units (f.f.u.) per ml.

Confirmation of virus inactivation. Inactivation of infectious virus in the HA antigen after acetone extraction was confirmed by inoculating the undiluted antigen (A) onto Vero E6 cells grown in a culture bottle. One week later, the cells were trypsinized and examined for infectious virus by IF staining. Part of the culture medium was further inoculated onto cells in the same way and the procedure was repeated.

Negative staining electron microscopy. The precipitate (P-2) prepared from the infected (B-1 strain) culture fluid was diluted with distilled water containing 0.1% glutaraldehyde and centrifuged at 25000 r.p.m. at 4 °C for 2 h in a Beckman 30 rotor. The supernatant was almost completely removed leaving only a small amount of the diluent, which was sonicated with the precipitate for 30 s and stained with 2% uranyl acetate. The sample was observed in a Hitachi model HU-12 electron microscope.

Test sera. Human and rat sera were used for the HI and IF tests. Human sera were (i) from a doctor who developed typical HFRS symptoms, taken periodically from day 3 to 30 after the onset of illness and (ii) five specimens from adults living in Osaka prefecture that gave HI-positive reactions against the B-1 strain in preliminary experiments. Rat sera were (i) 30 sera from rats that had been kept in an animal house polluted by HFRS virus and (ii) sera of rats that were immune to all four strains of HFRS virus. The preimmune status of these rats was proved to be negative to all the antigens by the HI test. The immune rat sera were collected 1 to 3 months after immunization and two different antisera were titrated for each strain of HFRS virus.

RESULTS

Properties of HA antigens

A re-precipitated sample of P-2 (Fig. 1) was observed by electron microscopy. Fig. 2 shows that the sample consisted of virus particles, many forming aggregates, with scarcely any cellular substances or other materials. Therefore, the HA activity of P-2 seemed to be due to the virions themselves. The virus particles were 80 to 130 nm in diameter (mean 110 nm) and were composed of an outer membrane and an inner nucleoid. In size and morphology, the virions of strain B-1 closely resembled those of other HFRS viruses (McCormick et al., 1982; White et al., 1982; Hung et al., 1983).

The HA activities and infectivities of samples at each step are summarized in Table 1. Although the starting material (F) showed an agglutination pattern of up to 16 just after the HA test, the pattern collapsed gradually and finally disappeared during overnight incubation. Almost all the virus particles were precipitated (P-2) by ultracentrifugation, leaving no infectious virus in the supernatant (S-2), and more than 50% of the particles of F were recovered. Acetone (A) and acetone-ether (AE) extractions did not increase the HA titre. However, acetone extraction inactivated the virus completely. Subsequent treatment by sonication for 3 min (AS-3) or 10 min (AS-10') increased the HA titre 2 or 4 times more. Moreover, the titres of these antigens increased by two- to fourfold on storage at −80 °C. These observations suggest that destruction of the virus particles is important for obtaining a high HA titre. Irrespective of the HA antigen used, the test sera showed the same HI titres. Therefore, AS-10', which had the highest titre, was used in the HI test.

Application of the HI test

To determine whether the HI test is suitable for diagnosis of HFRS, we compared this test with the IF test, which is at present the standard serological test for HFRS. Antigens from the B-1 strain were used in both tests. A patient with symptoms of HFRS was examined serologically
Fig. 2. Electron microscopic appearance of HA antigen (B-1 strain). The antigen was precipitated by ultracentrifugation and negatively stained with 2% uranyl acetate. Bar marker represents 100 nm.

Table 1. HA activity and infectivity of samples from each step in HA antigen preparation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (ml)</th>
<th>Titre</th>
<th>R* (%)</th>
<th>F.f.u./ml</th>
<th>R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>100</td>
<td>16 (&lt;2)†</td>
<td>100</td>
<td>5·0 x 10^5</td>
<td>100</td>
</tr>
<tr>
<td>S-1</td>
<td>100</td>
<td>16 (&lt;2)</td>
<td>100</td>
<td>4·2 x 10^5</td>
<td>84</td>
</tr>
<tr>
<td>P-1</td>
<td>2</td>
<td>128</td>
<td>16</td>
<td>2·8 x 10^6</td>
<td>11</td>
</tr>
<tr>
<td>S-2</td>
<td>100</td>
<td>&lt;2</td>
<td>&lt;13</td>
<td>&lt;10^3</td>
<td>&lt;0·02</td>
</tr>
<tr>
<td>P-2</td>
<td>2</td>
<td>1024</td>
<td>128</td>
<td>1·3 x 10^7</td>
<td>52</td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>1024</td>
<td>128</td>
<td>-‡</td>
<td></td>
</tr>
<tr>
<td>AE</td>
<td>2</td>
<td>1024</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS-3'</td>
<td>2</td>
<td>2048</td>
<td>256</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS-10'</td>
<td>2</td>
<td>4096</td>
<td>512</td>
<td></td>
<td></td>
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</table>

* Recovery rate.
† HA titre after overnight incubation at 4 °C.
‡ Infectious virus was completely inactivated as detected by the technique described in Methods.

(Fig. 3). The HI titres were lower than the IF titres, but the patterns of change in antibody determined by the HI and IF tests were parallel. Thus, both tests showed clearly that the patient was infected with HFRS virus.

Sera of rats that had been kept in an HFRS virus-polluted animal house were examined by the HI and IF tests, and the relation of the values is shown in Fig. 4. All the HI-positive sera also gave positive results in the IF test and 16 of 18 HI-negative sera gave negative results in the IF test. Two HI-negative sera gave positive results in the IF test, but their IF titres were very low. Antibody-negative and -positive sera were clearly differentiated by the HI test.

These data indicate that the results obtained by the HI test agreed well with those obtained by the IF test and that the IF test can be replaced by the HI test.
Fig. 3. Antibody titres in sera of a patient with HFRS determined by the HI (■) and IF (○) tests. The sera were taken periodically from 3 to 30 days after the onset of the illness.

Fig. 4. Comparison between HI and IF titres in sera of rats kept in an animal house polluted with HFRS virus.
Table 2. Comparison of HA antigens prepared from four different HFRS virus strains

<table>
<thead>
<tr>
<th>Sample</th>
<th>B-1</th>
<th>HV</th>
<th>PH</th>
<th>Hs</th>
</tr>
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<tbody>
<tr>
<td>P-2</td>
<td>1024</td>
<td>0</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>AS-10'</td>
<td>4096</td>
<td>128</td>
<td>256</td>
<td>1024</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>5.67</td>
<td>6.2</td>
<td>5.67-6.0</td>
<td>5.67-5.8</td>
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Table 3. HI antibody titres in human and rat sera against four HFRS virus strains

<table>
<thead>
<tr>
<th>Test serum</th>
<th>Patient (days after onset)</th>
<th>B-1</th>
<th>HV</th>
<th>PH</th>
<th>Hs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>640</td>
<td>320</td>
<td>640</td>
<td>160</td>
</tr>
<tr>
<td>Human (no.)</td>
<td>1</td>
<td>10</td>
<td>20</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>40</td>
<td>80</td>
<td>160</td>
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<td>80</td>
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<tr>
<td></td>
<td>5</td>
<td>160</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Rat (no.)</td>
<td>1</td>
<td>2560</td>
<td>80</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>≤10240</td>
<td>40</td>
<td>1280</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1280</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>320</td>
<td>40</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Immune rat</td>
<td>Anti-B-1 (1)</td>
<td>1280</td>
<td>160</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>1280</td>
<td>80</td>
<td>40</td>
<td>160</td>
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<tr>
<td></td>
<td>Anti-HV (1)</td>
<td>80</td>
<td>1280</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>80</td>
<td>640</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Anti-PH (1)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>640</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>640</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>Anti-Hs (1)</td>
<td>20</td>
<td>80</td>
<td>80</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>20</td>
<td>80</td>
<td>80</td>
<td>320</td>
</tr>
</tbody>
</table>

Comparison of HA antigens prepared from four HFRS virus strains

HA antigens of three other strains (HV, PH, Hs) were prepared in the same way as that of the B-1 strain, and their properties are summarized in Table 2. Before the acetone and sonication treatment of P-2, only the B-1 strain had a high titre. But after this treatment (AS-10') all the strains showed high HA titres, and this treatment was essential for obtaining HA antigen from the HV strain. The difference in the HA titres of the strains did not reflect differences in the properties of the strains, but merely fluctuations in the conditions of virus propagation, because in another experiment even P-2 of HV showed a high HA titre. No significant differences were found in the optimum pH values for antigenicity of the strains; with increase in pH the HA titres decreased, and at pH 7.0, no agglutination pattern was observed with any strain. With all the virus strains, HI tests were performed at pH 5.8.

HI antibody titres in human and rat sera against four HFRS virus strains

Table 3 shows the HI antibody titres of test sera against the four HFRS virus strains. Human sera, including sera from a patient, showed strong cross-reactivity with all the strains. On the other hand, rat sera showed significantly higher titres against the B-1 strain, suggesting that the rats were infected with viruses related to the B-1 strain. Rat sera immunized with one of the four
different strains showed the highest titres to the homologous virus. In particular, anti-PH rat sera showed high specificity for the homologous virus.

**DISCUSSION**

The HI test is easy to perform, reliable and objective, but it has scarcely ever been used for serological diagnosis of HFRS. Tsai *et al.* (1984) reported the use of the HI test with antigen prepared from SMB for identification of HFRS. Usually, SMB is the best source of the HA antigen of bunyaviruses, the group to which HFRS virus belongs (Schmaljohn *et al.*, 1985). However, in the case of HFRS virus, SMB does not seem to be an appropriate antigen source, because the HA titre is not high enough (1:16). Moreover, as HFRS infection may occur on inhaling air polluted with the virus, homogenization of SMB may be dangerous.

The HA antigen of the B-1 strain prepared in this work from infected culture fluid was easy to obtain and had a high titre (1:4096). Interestingly, infected culture fluid showed agglutination for only a short period (Table 1). The reason for this is unknown. When the antigen was precipitated by ultracentrifugation and suspended in BABS pH 9, it was stable with no change of its agglutination pattern for a long period, and when it was kept at 4 °C its HA activity did not change for at least 3 weeks. Acetone extraction completely inactivated the infectious virus in the antigen preparation and thus makes its use in any laboratory possible.

Ardoin & Clarke (1967) reported that sonication markedly increases the HA titre of group C arboviruses, which are now classified in the family Bunyaviridae. We observed the same phenomenon with HFRS virus, especially the HV, PH and Hs strains (Table 2). The marked increase of the HA titre on acetone-sonication treatment seemed to be due to dissociation of agglutinated virus particles and/or release of virus components with HA activity from the virus envelope. Furthermore, we tried to increase the HA titre by increasing the NaCl concentration in the viral adjusting diluent (VAD) from 0.15 M to 0.4 M as suggested by Beaty *et al.* (1977) who showed that HA titres of antigens from nine serological groups of Bunyaviridae were increased by hypertonic NaCl. Although some HA antigens increased in titre by two- or fourfold in the VAD with 0.4 M-NaCl, incomplete RBC sedimentation made it difficult to determine the endpoints of the HA titres. Therefore, high salt concentration in the VAD does not seem to have any advantages over normal salt concentration for the HFRS virus HA antigens.

In preliminary experiments, the HA test was performed at 37 °C, room temperature and 4 °C. At each temperature, the HA titre was the same for all antigen preparations. The reaction period and its temperature were also studied in the HI test for HA antigen and test serum. In any condition (37 °C, 1 h; room temperature, 1 h; 4 °C, overnight), no significant differences in the HI titres of the test sera were observed.

The considerable cross-reaction of the HFRS virus strains in the HI test, especially with human sera (Table 3), indicates that these strains constitute one serogroup. This is the first report demonstrating by the HI test that there are antigenic similarities among HFRS viruses isolated in different areas in the world. However, sera from experimentally immunized rats showed type-specific HI patterns with slight cross-reactivity to the heterologous HFRS virus strains. Franko *et al.* (1983) reported that monoclonal antibodies to Hantaan virus reacted monospecifically to the homologous Hantaan virus in an indirect IF test. In our own recent study using monoclonal antibodies to the HFRS viruses, some antibodies showed high HI titres only against homologous virus without showing any detectable HI titres against heterologous viruses; some antibodies showed high HI titres to all the HFRS virus strains. These observations suggest that HFRS virus has two, type-specific and group-reactive, antigenic determinants which are responsible for HA activity. In this case, in the HI test (Table 3), strong cross-reactivity in the human sera is mainly due to antibodies induced by group-reactive antigenic determinants, whereas type specificity in the rat sera, especially anti-PH sera, is mainly due to antibodies induced by type-specific antigenic determinants.

Although the insights gained from the HI test are limited at the present time, the test has possibilities for use not only for serodiagnosis of HFRS but also for analysis of antigenic differences among HFRS virus strains.
We express our sincere thanks to Mr T. Wada for his excellent technical help in electron microscopy.

REFERENCES


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