Definition of Three Serotypes of Hantaviruses by a Double Sandwich ELISA with Biotin–Avidin Amplification System

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(Accepted 18 April 1985)

SUMMARY

A total of seven different isolates of Hantaviruses from five mammalian species and six geographical areas were examined by a double sandwich ELISA with biotin–avidin amplification system. Three serotypes were recognized by comparing antigen titres to homologous and heterologous antibody and blocking antibody titres to homologous and heterologous antigens. Serotype 1 included four strains: Hantaan virus isolated from Apodemus agrarius in South Korea, Hupei-I isolated from acute-phase serum from a patient with epidemic haemorrhagic fever (EHF) in China, SR-11 isolated from a laboratory rat associated with an EHF outbreak in Sapporo, Japan, and Tchoupitoulas isolated from a wharf rat (Rattus norvegicus) trapped in New Orleans, La., U.S.A. Serotype 2 included two strains of nephropathia epidemica virus, Hällnäs-I and Puumala, isolated from Clethrionomys glareolus trapped in nephropathia epidemica foci in Sweden and Finland, respectively. Serotype 3 included one strain, Prospect Hill-I, isolated from Microtus pennsylvanicus, trapped in Frederick, Md., U.S.A.

INTRODUCTION

Hantaan virus, isolated from Apodemus agrarius in 1977, is the prototype of viruses causing haemorrhagic fever with renal syndrome (HFRS) (Lee & Lee, 1976). Electron micrographs of the virus, its protein composition, its tripartite single-stranded RNA genome and the RNA 3' terminal sequence, indicate a close resemblance to Bunyaviruses (White et al., 1982; McCormick et al., 1982; Schmaljohn & Dalrymple, 1983; Elliott et al., 1984). The serological relatedness of Korean haemorrhagic fever (KHF), epidemic haemorrhagic fever (EHF) in China, HFRS in the U.S.S.R. and nephropathia epidemica (NE) in Scandinavia has been well documented (Lee, 1982; Gajdusek et al., 1983). A number of virus strains antigenically related to Hantaan virus have been isolated from HFRS patients as well as from rodents associated or unassociated with recognized HFRS outbreaks in different regions of the world (Brummer-Korvenkontio et al., 1980; Lee et al., 1982b; LeDuc et al., 1982; Tsai et al., 1982; Song et al., 1982; Gavrilovskaya et al., 1983; Kitamura et al., 1983; Yamanishi et al., 1983; Yanagihara et al., 1984; Niklasson & LeDuc, 1984). Several serological methods have been employed in the study of these isolates, but virus serotypes have not yet been defined. The recent publication of a highly sensitive immunofluorescence technique utilizing biotin–avidin interaction to detect herpes simplex virus (Nerurkar et al., 1983) stimulated us to develop an ELISA with biotin–avidin amplification system for the Hantaviruses. We now report the definition of Hantaan virus serotypes using this technique.

METHODS

Cell culture. The Vero C1008 clone of Vero cells (Vero E6 cells) was purchased from the American Type Culture Collection (cat. no. ATCC C1008, CRL 1586). Cell cultures were grown at 37 °C in a 5% CO₂ incubator with 90% humidity in Eagle’s minimal essential medium supplemented with 5% heat-inactivated foetal bovine serum, 2 mM-L-glutamine and gentamicin.

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Viruses. The Hantaan virus strain 76-118 was isolated from Apodemus agrarius trapped in the KHF endemic focus in South Korea (Lee & Lee, 1976). The strain Hupei-I was isolated in our laboratory from an early-phase serum of a Chinese patient with EHF (Hsiang et al., 1984). The SR-11 strain, recovered from a laboratory rat and associated with an EHF outbreak in Sapparo, Japan (Kitamura et al., 1983), was provided by Dr K. Sugiyama, National Institutes of Health, Japan. Tchoupitoulas virus, isolated from a wharf rat, Rattus norvegicus, trapped in New Orleans, La., U.S.A. (Tsai et al., 1982) was provided by Dr T. Tsai, Division of Vector-Borne Viral Diseases, C.D.C., Atlanta, Ga., U.S.A. Two strains of NE virus, Hällnäs-I, isolated in our laboratory (Yanagihara et al., 1984) and Puumala (Brummer-Korvenkontio et al., 1980) obtained from the American Type Culture Collection, were recovered from Clethrionomys glareolus trapped in NE endemic foci in Sweden and Finland, respectively.

Antigen preparation. Infected or mock-infected Vero E6 cells were scraped from flasks with a rubber policeman and then washed in cold phosphate-buffered saline (PBS), pelleted, resuspended to a concentration of 10^8 in Tris-HCl buffer pH 7.4, containing 0.1 M-NaCl and 0.002 M-EDTA, and solubilized by treatment with 1% NP40 (Sigma) (final concentration) for 15 min at 4 °C. Nuclei were spun down at 3000 g for 20 min and supernatants were used as antigens. The protein concentration of all antigen preparations was adjusted to 6 mg/ml, as measured by the Bio-Rad Protein Assay.

Antisera. Immune sera to virus strains 76-118, Hupei-I, Tchoupitoulas, and SR-11 were produced in Fisher laboratory rats (F344/N strain) by intramuscular inoculation of live viruses grown in Vero E6 cells. Sera were collected 6 weeks later. Antiserum to Hällnäs-I was made in a cynomolgus monkey inoculated intravenously with a 10^6 homogenate of virus-infected C. glareolus lung; antiserum to Prospect Hill virus was made in a cynomolgus monkey inoculated with medium of Prospect Hill virus-infected Vero E6 cells (Amyx et al., 1984). Monkey sera were collected 6 months after inoculation. In addition, a convalescent-phase serum from a NE patient was included in the tests.

Preparation of capturing antibody (Abc). IgG isolated from antisera by DEAE–Sephacel (Pharmacia P-L Biochemicals) chromatography according to the manufacturer's instructions was used as capturing antibody. Isolated IgG was mixed 1:1 with glycerol, and stored at 4 °C.

Biotinylated antibody (Abb) preparation. The globulin fraction obtained from antiserum by ammonium sulphate precipitation was conjugated to biotin as described by Nerurkar et al. (1983). The biotinylated protein was separated from unbound biotin and dimethyl sulphoxide by gel filtration on Sephadex G-50 (PD-10 column, Pharmacia P-L Biochemicals), divided into aliquots and stored at -70 °C.

Double sandwich ELISA with biotin–avidin amplification system

Antigen titration. The double sandwich ELISA was based on a radioimmunoassay for hepatitis B virus (Purcell et al., 1973), with modifications. Costar 96-well polyvinylchloride flexible plates (cat. no. 2596) were used in this study. The flow chart of the test, with six stages, is shown in Table 1. Each of five viral antigens, Hantaan, SR-11, Hupei-I, Hällnäs-I, and Prospect Hill-I were titrated against homologous antibody (both Abc and Abb prepared from homologous antiserum) as well as against heterologous antibodies (both Abc and Abb prepared from heterogeneous antiserum).
### Table 2. Antigen titres of Hantaviruses determined by a double sandwich ELISA with biotin–avidin amplification system

<table>
<thead>
<tr>
<th>Viruses</th>
<th>KHF Hantaan</th>
<th>Sapporo rat SR-11</th>
<th>Louisiana rat Tchoupitoulas</th>
<th>EHF Hupei-I</th>
<th>NE Hällnäs-I</th>
<th>Puumala</th>
<th>Prospect Hill-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHF Hantaan</td>
<td>64*</td>
<td>64</td>
<td>8</td>
<td>256</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sapporo rat SR-11</td>
<td>64</td>
<td>64</td>
<td>8</td>
<td>256</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EHF Hupei-I</td>
<td>64</td>
<td>128</td>
<td>8</td>
<td>128</td>
<td>128</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>NE NEh∞†</td>
<td>-</td>
<td>64</td>
<td>32</td>
<td>128</td>
<td>16 128</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>Hällnäs-I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16 128</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Prospect Hill-I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 64</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* The titres are the reciprocal of highest antigen dilutions causing 2.1-fold increase in absorbance at 415 nm. Titres of less than 4 are shown as -.
† Capturing and biotinylated antibody prepared from NE patient serum.

RESULTS

**Antigen titration**

Illustrative data showing the antigenic relationship among seven Hantaviruses when capturing and biotinylated antibodies to individual virus strains were used to titrate homologous and heterologous viral antigens are summarized in Table 2. Four strains, Hantaan 76-118, Hupei-I, SR-11 and Tchoupitoulas, were found to be antigenically closely related to each other. In contrast, the two strains of NE virus, Puumala and Hällnäs-I, both isolated from C. glareolus trapped in Scandinavia, were antigenically distinct from other Hantaviruses and were recognized only by antibody in human convalescent-phase serum following NE infection or by anti-Hällnäs antibody prepared in monkeys. Anti-Prospect Hill virus antibody detected only homologous antigen. One-way cross-relationships between NE and Prospect Hill virus strains as well as between NE virus strains and the Hantaan-related group of four viruses were detected. The human convalescent serum containing antibodies to NE virus (NEh∞) had a broader spectrum of cross-reactivity than the monkey anti-Hällnäs-I antibody, and reacted with all antigens except Hantaan virus antigen, while the anti-Hällnäs-I monkey antibody detected only Hällnäs-I, Puumala and Hupei-I antigens.
Table 3. *Blocking antibody titres to Hantaviruses determined by a double sandwich ELISA with biotin–avidin amplification system*

<table>
<thead>
<tr>
<th>Test viruses (antigen, Ab&lt;sub&gt;1&lt;/sub&gt; and Ab&lt;sub&gt;2&lt;/sub&gt;)</th>
<th>Origin of Hantavirus</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td>Geographical location</td>
</tr>
<tr>
<td>KHF Hantaan</td>
<td>A. agrarius</td>
<td>South Korea</td>
</tr>
<tr>
<td>Sapporo rat SR-11</td>
<td>Laboratory rat</td>
<td>Sapporo, Japan</td>
</tr>
<tr>
<td>EHF Hupei-1</td>
<td>Human</td>
<td>Hupei, China</td>
</tr>
<tr>
<td>NE Hällnäs-I (NE&lt;sub&gt;s&lt;/sub&gt;)†</td>
<td>C. glareolus</td>
<td>Västerbotten</td>
</tr>
<tr>
<td>Hällnäs-I</td>
<td>C. glareolus</td>
<td>County, Sweden</td>
</tr>
<tr>
<td>Prospect Hill-I</td>
<td>M. pennsylvanicus</td>
<td>Maryland, U.S.A.</td>
</tr>
</tbody>
</table>

* The titres are the reciprocal of highest antiserum dilutions causing 50% reduction in absorbance at 415 nm. Titres of less than 16 are shown as –.
† Hällnäs-I virus antigen; capturing and biotinylated antibody prepared from NE patient serum.
Serotypes of Hantaviruses

Table 4. Serotypes of Hantaviruses defined by a double sandwich ELISA with biotin–avidin amplification system

<table>
<thead>
<tr>
<th>Serotype 1</th>
<th>Korean haemorrhagic fever</th>
<th>Hantaan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sapporo rat</td>
<td>SR-11</td>
</tr>
<tr>
<td></td>
<td>Louisiana rat</td>
<td>Tchoupitoulas</td>
</tr>
<tr>
<td></td>
<td>Epidemic haemorrhagic fever</td>
<td>Hupei-I</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serotype 2</th>
<th>Nephropathia epidemica</th>
<th>Hällnäs-I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prospect Hill-I</td>
<td>Puumala</td>
</tr>
</tbody>
</table>

| Serotype 3 | Prospect Hill             | Prospect Hill-I |

Blocking antibody titrations

The homologous and heterologous antigenic cross-reactions among Hantaviruses based on blocking antibody titrations are shown in Table 3. Abh, Abc and homologous antigen of each strain were used to titrate the blocking capacity of all antiviral antisera. The blocking effect observed with the Hantaan, Hupei-I, SR-11, and Tchoupitoulas antisera revealed a close relationship between these strains. Anti-Hällnäs-I and anti-Prospect Hill-I antisera blocked only homologous antigens, thus confirming that these two viruses are antigenically distinct from one another as well as being distinct from the other strains of Hantaviruses tested. Blocking antibody titration showed no cross-reactions among the three groups of viruses.

Based on the antigenic cross-reactions between seven strains of Hantaviruses as determined by double sandwich ELISA employing biotin–avidin amplification, three major serotypes have been recognized as shown in Table 4. They are serotype 1, composed of Hantaan, SR-11, Tchoupitoulas and Hupei-I viruses; serotype 2, composed of Hällnäs-I and Puumala viruses; and serotype 3, composed of Prospect Hill-I virus.

DISCUSSION

The double sandwich ELISA described for use in the study of Hantaviruses was based on a radioimmunoassay protocol described for hepatitis B virus (Purcell et al., 1973). The successful application of the biotin–avidin interaction in immunochemistry and immunofluorescence (Bayer & Vilchek, 1980) together with the introduction of avidin–biotin–enzyme complexes (Hsu et al., 1981) has led to the development of a double sandwich ELISA with biotin–avidin amplification system. In this study, biotin–antibody conjugates prepared by a simple and reliable two-step procedure (Nerurkar et al., 1983) have both higher sensitivities and lower backgrounds than horseradish peroxidase–antibody conjugates prepared by the method of Nakane (1980). Biotinylated antibodies prepared from globulin fractions precipitated by ammonium sulphate or from IgG purified by ion-exchange chromatography worked equally well (unpublished data). Serum with an antibody titre as low as 1 : 1000 was suitable for the preparation of Abh and Abc, and ABC complexes prepared from commercially available avidin and biotinylated enzyme proved to be an excellent amplification system. The high affinity of the avidin and biotin interaction (dissociation constant 10^{-15} M) and the formation of multiple array complexes due to the presence of four biotin-binding sites on each avidin molecule (Hsu et al., 1981) contribute to both the high specificity and sensitivity of the ELISA described (Yolken et al., 1983).

In the present study we have compared seven strains of Hantavirus isolated from five mammalian species collected in six geographical areas. Three serotypes were recognized on the basis of a difference between homologous and heterologous reciprocal antigen titres as well as by the endpoint titres of blocking antibodies. Serotype 1 is composed of four closely related strains and includes Hantaan virus, the SR-11 and Tchoupitoulas strains of rat viruses, and the Hupei-I strain isolated from a patient in China. Serotype 2 includes Hällnäs-I and Puumala viruses both isolated from C. glareolus trapped in endemic foci of Scandinavia. Serotype 3 includes Prospect Hill-I virus isolated from a Micromys muscle. The four strains of viruses belonging to serotype 1 cross-reacted with each other completely, yet, as noted above, these viruses were isolated from samples of three mammalian species (A. agararius, R. norvegicus and man) in four geographical
areas (South Korea, China, Japan and the United States). Two of these four strains, SR-11 and Tchoupitoulas (both isolated from rats), were antigenically indistinguishable in the tests. Both were recognized by serotype 1 antibody and by antibody in the convalescent serum of a NE patient. Hupei-I antigen also reacted with antibody to NE virus prepared in monkeys. In contrast, SR-11 and Tchoupitoulas antigens were not detected by antibodies experimentally induced in cynomolgus monkeys by either Hålñäs-I or Prospect Hill-I viruses. Hantaan antigen was recognized only by serotype 1 antibody. These data thus suggest that although the viruses belonging to serotype 1 are closely related strains of Hantaviruses, they are not antigenically identical. The Hålñäs-I and Puumala antigens were detected by anti-Hålñäs antibody and convalescent NE patient antibody exclusively. Finally, Prospect Hill-I virus antigen was detected not only by homologous antibody but also by antibody in the convalescent NE patient’s serum (Table 2).

The tests thus revealed one-way cross-reactions of serotype 2 antibodies with the serotype 3 antigen as well with some of the serotype 1 antigens. Titrations of the blocking capacities of the antisera delineated the three serotypes even more distinctly; none of the antisera of one serotype was able to block the antigen of another serotype (Table 3). Virus nucleocapsid protein is apparently responsible for the reaction with polyclonal antisera to Hantaviruses in ELISA (our unpublished results).

Our results largely confirm previous conclusions regarding the serological differentiation of various Hantaviruses, based on indirect immunofluorescence (IF) tests, ELISA or radioimmunoassay (RIA). Svedmyr et al. (1979, 1980, 1982) and Lee et al. (1980, 1982a, b) using Hantaan and NE viruses and patient antisera in IF tests noted the relatedness of KHF, EHF, HFRS, and NE as well as the antigenic distinctiveness of KHF and NE. No virus antigen was detected by IF on the surface of the cells infected with Hantaviruses. Chumakov & Gavrilovskaya (1980) and Tkachenko et al. (1982) using ELISA and RIA with pools of wild rodent lung suspensions as antigen and HFRS patient sera as the source of capturing antibody, confirmed the difference in reactivity between some convalescent HFRS patient sera from the European and Far Eastern parts of the U.S.S.R. A relative difference in antibody titre was demonstrated by IF tests when various HFRS patient and animal sera were titrated against Hantaan, NE and Prospect Hill antigens (Lee et al., 1984). Analyses of antiviral antibody by RIA and plaque reduction neutralization tests showed that Hantaan, SR-11 and Tchoupitoulas strains are antigenically very close to each other but different from both Puumala and Prospect Hill viruses (Schmaljohn et al., 1983). A serological comparison by ELISA of the antigens of two viruses related to Hantaan virus (one isolated from A. agrarius and the other from R. norvegicus) was recently published by Chinese investigators who found the strains to be similar, but different (Song et al., 1984). Japanese investigators using immune adherence haemagglutination demonstrated a similar antigenic relationship between Hantaan virus isolated from A. agrarius and three virus strains, SR-11, TSR 352 and Tchoupitoulas isolated from rats (Sugiyama et al., 1984b).

Analysis of further isolates, from various sources, and the use of a variety of serological methods will be required for a complete serological classification of Hantaviruses.

We thank Dr David M. Asher for the helpful criticism of the work. We also thank Mrs Linda Poole and Mrs Ginny Rousculp for their assistance in the preparation of the manuscript.

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(Received 31 December 1984)