Hantaan Virus: Identification of Virion Proteins

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(Accepted 18 April 1984)

SUMMARY

SDS–PAGE and immunoprecipitation analyses were carried out on the virion and cell-associated proteins of Hantaan virus, the causative agent of haemorrhagic fever with renal syndrome (HFRS). Purified virions have a density of 1.17 g/ml in sucrose, and contain four proteins with molecular weights of 45000 (45K), 56K, 72K and 200K, confirming recent evidence that the virus is a member of the family Bunyaviridae. Detergent treatment of virions indicates that the 45K protein is the virus nucleoprotein. Both the 72K and the 56K proteins were labelled with [3H]glucosamine and were removed from virions by bromelain treatment, indicating that they are envelope glycoproteins. The 200K protein was found only in [35S]methionine-labelled preparations. By analogy to prototype viruses of the family Bunyaviridae, these proteins were designated N, G1, G2, and L respectively. Three virus-specific proteins (N, G1, G2) were detected in virus-infected cells. These proteins were precipitable by human convalescent serum and by serum of a Rattus norvegicus trapped in the United States. No additional virus proteins were detected in infected cells. These results confirm recent morphological and RNA studies that Hantaan virus is a member of the family Bunyaviridae. Our results also support the suggestion that Hantaan virus be placed in a new genus of Bunyaviridae.

INTRODUCTION

The disease haemorrhagic fever with renal syndrome (HFRS) gained attention in 1951 when many United Nations military personnel contracted the disease in Korea (Smadel, 1953). It was not until 1978 that a viral agent, Hantaan virus, was isolated from Apodemus agrarius coreae, a rodent reservoir (Lee et al., 1978). On the basis of virion morphology (McCormick et al., 1982; White et al., 1982) and virion RNA properties (Schmaljohn & Dalrymple, 1983; Schmaljohn et al., 1983), it has been proposed that Hantaan virus be classified as a member of the family Bunyaviridae. We recently adapted Hantaan virus to replicate in the E6 clone of Vero 76 cells (McCormick et al., 1982). The increased sensitivity and yield of these cells has allowed the isolation of additional virus strains and more advanced morphological and biochemical studies (Tsai et al., 1982a; Kitamura et al., 1983; Hung et al., 1983). The virus particles are spherical with an average diameter of 92.5 nm, with a lipoprotein envelope and capsomeres which give them a knobby appearance (McCormick et al., 1982). In this paper, we report the characterization by SDS–PAGE of four proteins which appear to be analogous to the L, G1, G2, and N proteins of prototype Bunyaviridae. The molecular weights of these proteins, however, differ from those of other members of this family.

Hantaan virus represents a significant biohazard and has been assigned to Biosafety Level 4 in the U.S. Public Health Service’s guidelines. Our work was conducted in the Maximum Containment Laboratory (MCL) at the Centers for Disease Control (CDC).

METHODS

Cell cultures. The E6 cell line, a cloned line of Vero cells (ATCC Vero clone CRL 1586), was used in these studies (McCormick et al., 1982; Johnson et al., 1981). Cells were grown in Eagle’s minimum essential medium (MEM)

00022-1317/84/0000-6027

Key words: Hantaan virus/bunyaviruses/protein characterization
supplemented with 10% heat-inactivated foetal bovine serum (FCS) and an antibiotic mixture of 100 units of penicillin, 50 μg of streptomycin, and 2 μg of amphotericin B per ml. Cells were free of mycoplasma. After inoculation with virus, growth medium was replaced with maintenance medium in which the FCS was reduced to 2%.

Growth and purification of the virus. Hantaan virus strain 76–118 was used in these studies (Lee et al., 1978; McCormick et al., 1982; French et al., 1981). Virus stocks were prepared from a plaque-purified seed virus preparation by infection of Vero E6 cells at an m.o.i. of 0·1 p.f.u./cell. After 7 days, the supernatant fluid was clarified by centrifugation at 2000 r.p.m. for 15 min. Bovine serum albumin (BSA) was added to 1·5% before freezing at −70°C. Virus preparations had an infectivity titre of 1 × 10^7 p.f.u./ml.

For production of purified virus, supernatant fluids from twenty 150 cm² flasks, infected for 7 days, were clarified by centrifugation at 3000 r.p.m. for 15 min and layered onto a 20% sucrose cushion for centrifugation in a Spinco SW27 rotor at 22000 r.p.m. for 1 h. Larger volumes could be pelleted in the Beckman J-21 centrifuge at 8000 r.p.m. overnight (H. Lindsey-Regnery, personal communication). Pellets were suspended in sterile Tris–NaCl–EDTA (TNE) buffer, layered on a 20 to 70% sucrose gradient and centrifuged to equilibrium for 18 h at 30000 r.p.m. in the Spinco SW40 rotor. The virus material appeared as an opalescent band at a density of 1·17 g/ml. This band was harvested, diluted in TNE and pelleted at 35000 r.p.m. for 1 h in the SW40 rotor. The pellet was suspended in 200 μl of TNE and frozen at −70°C.

If the virus was to be radioactively labelled, the maintenance medium was replaced on day 4 with labelling medium consisting of 20% MEM, 2% dialysed FCS, 80% specific nutrient-deficient MEM to which radioactive nutrients were added as follows: 2 μCi/ml of [14C]amino acids, 20 μCi/ml of D-[6-3H]glucosamine, 10 μCi/ml of [3H]leucine, or 20 μCi/ml of [35S]methionine (all from New England Nuclear). Supernatant fluids were collected on day 7 and purified as described above.

Immunoprecipitation of viral proteins. The radioimmunoprecipitation (RIP) procedure used was essentially that described by Kessler (1975). E6 cells in 25 cm² flasks were infected with Hantaan virus at an m.o.i. of 1. After an adsorption period of 30 min, maintenance medium was added and the cells incubated for the indicated times at 37°C. Three h before harvesting the cells, the maintenance medium was replaced with methionine-free MEM.

One h later this medium was removed and replaced with methionine-free MEM containing 50 μCi/ml of [35S]methionine. After a 2 h labelling period, medium was removed, cells were washed twice with cold, sterile phosphate-buffered saline (PBS), and then 1 ml of RIP buffer per 25 cm² flask was added. RIP buffer consisted of NET buffer (50 mM-Tris-HCl, 0·15 M-NaCl, 5 mM-EDTA, 0·02% NaN₃) with 1% Nonidet P40, 0·5% deoxycholate (DOC), and 0·1% SDS. Just before use, 1 mM-phenylmethylsulphonyl fluoride (PMSF) was added.

After 15 min at 4°C, the infected cell extract was placed in a 1·5 ml Eppendorf centrifuge tube, mixed well to shear the DNA, and then centrifuged for 2 min in a Beckman Microfuge. 100 μl of the resultant cell extract was mixed with 20 to 50 μl of immune serum and incubated at room temperature for 2 h. Unless otherwise indicated, our standard antiserum was mouse ascites fluid (MAF) no. 701469 which was prepared in our laboratory against Hantaan strain 76–118 and has an indirect fluorescent antibody (IFA) titre of 1:2048. 100 μl of a 10% suspension of staphylococcal Protein A–Bacterial Adsorbent (SPA, Mfles-Yeda, Ltd.) was then added, and this mixture was incubated at room temperature for 15 to 30 min with frequent vortex-mixing. The SPA–antigen–antibody complex was pelleted, washed twice with cold NET buffer containing 0·1% NP40 and 0·1% DOC, and pelleted proteins were dissociated by boiling for 2 min in 100 μl of NET buffer containing 2% SDS, 2% 2-mercaptoethanol, 50% sucrose and bromophenol blue (BPB).

SDS–PAGE. The procedure used was that described by Laemmli (1970) except that 2.5 M-urea was incorporated into the gels. The separating gels contained 15% or 12.5% acrylamide and the stacking gels contained 3% acrylamide. Slab gels measured 1·5 mm × 14 cm × 20 cm and were electrophoresed at a constant voltage of 50 V for 16 h. Gels were fixed in 10% acetic acid for 1 h, placed in EN3HANCE (New England Nuclear) for 1 h, rinsed in water for 30 min, dried under vacuum and exposed to X-ray film (Kodak X-Omatic or DuPont Cronex 4) at −70°C. Appropriate molecular weight standards were included. 12.5% acrylamide cylindrical gels were also run at 50 V for 16 h. After electrophoresis, gels were extruded into 10% acetic acid for 1 h, then frozen and sliced into 1 mm sections. Slices were placed in 6 ml of 4a20 (Research Products International, Elk Grove Village, Ill., U.S.A.) scintillation fluid containing 6% TS-1 tissue stabilizer and incubated at 37°C overnight. Radioactivity of each sample was determined in a Beckman scintillation counter.

RESULTS

SDS–PAGE analysis of purified virion proteins

Three Hantaan virion proteins with estimated molecular weights of 45000 (45K), 56K and 72K were detected by SDS–PAGE analysis with Coomassie Brilliant Blue staining and radioactive labelling (Fig. 1). When 35S-labelled virion proteins were electrophoresed, a fourth
Hantaan virus proteins

Fig. 1. Electrophoretic analysis of Hantaan virion proteins. Hantaan virus grown in the presence of 
\(^{14}\)C-amino acids was purified, disrupted and proteins were separated in a 12.5\% polyacrylamide gel. (a) 
Prestained mol. wt. standards (Bethesda Research Laboratories) consisting of, in order of decreasing 
size, myosin (H chain), phosphorylase b, bovine serum albumin, ovalbumin and \(\alpha\)-chymotrypsinogen; 
(b) extracellular virus protein after pelleting through a 20\% sucrose cushion, Coomassie Brilliant Blue 
stain; (c) virus from (b) following further purification in a 20 to 70\% sucrose gradient, Coomassie 
Brilliant Blue stain; (d) autoradiograph of preparation used in (c).

protein with a mol. wt. of approximately 200K was observed (Fig. 2a, c and Fig. 6). These four 
proteins correspond, respectively, to the N, G1, G2, and L proteins reported for prototype 
member viruses of the family *Bunyaviridae* (Obijeski & Murphy, 1977).

Virion glycoproteins

When the virus was grown in the presence of \(^{3}\)H]glucosamine the 72K and 56K proteins were 
labelled (Fig. 2b). These are analogous to the G1 and G2 glycoproteins of members of the family 
*Bunyaviridae*. To further characterize these glycoproteins, purified \(^{35}\)S-labelled particles were 
treated with bromelain (Obijeski *et al.*, 1976), and then pelleted at 35000 r.p.m. for 2 h in an 
SW50.1 rotor. Fig. 2(e) shows that the glycoproteins were removed by this treatment and that 
only the L and N proteins remained associated with virions.

Identification of the virion nucleocapsid protein

If Hantaan virus is a member of the family *Bunyaviridae*, then one of its proteins should be 
associated with a helical nucleocapsid (Obijeski & Murphy, 1977). To test this premise, virus-
infected cells were double-labelled with \(^{3}\)H]glucosamine and \(^{14}\)C-amino acids 5 to 7 days post-
fection. Virus was partially purified through a 20\% sucrose cushion, then treated with NET 
plus 1\% NP40 for 15 min at 4°C and re-pelleted (Lesnaw & Dickson, 1978). Because the virus 
was only partially purified by this procedure, RIP was used to concentrate and further purify 
virus proteins. When this was done, only the 45K protein was associated with the pelleted virion 
nucleocapsid fraction (Fig. 3). Normal MAF precipitated only non-viral, cellular proteins. 
Studies in this laboratory using monoclonal antibodies (unpublished results) have confirmed 
that the 45K protein is the nucleocapsid protein.
Fig. 2. Identification of Hantaan virus proteins by SDS–PAGE. (a) $^{[35S]}$Methionine-labelled Hantaan virus, purified through a 20% sucrose cushion and a 20 to 70% sucrose gradient. The pellet was resuspended in NET buffer with 2% SDS, 2% 2-mercaptoethanol, 50% sucrose, and BPB, boiled 2 min and electrophoresed on a 12.5% acrylamide tube gel. The gel was cut into 1 mm slices and radioactivity was estimated. (b) One 150 cm$^2$ flask of E6 cells was infected for 7 days and labelled for 72 h with 50 μCi/ml of $[^3H]$glucosamine. The supernatant was centrifuged through a 20% sucrose cushion and the pellet processed as in (a). (c) $^{35S}$-labelled Hantaan virus treated with bromelain at 37 °C for 30 min, pelleted at 35000 r.p.m. for 2 h, and electrophoresed in a tube gel as in (a).

Fig. 3. Identification of virion nucleocapsid proteins. Virus was double-labelled with $[^3H]$glucosamine and $^{14}$C-amino acids 5 to 7 days post-infection and partially purified through 20% sucrose. Samples in (a) and (b) were treated with NET buffer with 1% NP40 only. Samples in (c) and (d) were treated with RIP buffer. (a) Pellet from NP40-treated sample, RIP with MAF 701469; (b) supernatant from this. (c) Sample treated with RIP buffer and RIP with MAF 701469; (d) same sample as (c) but RIP with normal MAF.

Intracellular proteins

In the experiment depicted in Fig. 4, cells were labelled for 2 h with $^{[35S]}$methionine at 24, 48, and 72 h post-infection. Sera used for immunoprecipitation were convalescent human sera from survivors of HFRS (no. 700047 and 126462). Both sera had IFA titres of 1:1024. The rodent
Fig. 4. Time course of protein synthesis. Radioimmunoprecipitation of Hantaan virus-infected E6 cells, labelled for 2 h with [35S]methionine and run on 15% acrylamide gels without urea. (a to c) 24, 48, 72 h cell extracts RIP with Baltimore rat no. 5 serum; (d to f) 24, 48, 72 h cell extracts RIP with human serum no. 700047; (g to i) 24, 48, 72 h cell extracts RIP with human serum no. 126462; (j) 72 h cell extract RIP with normal human serum; (k) uninfected E6 cell extract RIP with human serum no. 700047; (l) uninfected E6 cell extract RIP with Baltimore rat no. 5 serum.

Table 1. Comparison of Hantaan virion-associated proteins with those of four prototype viruses of the family Bunyaviridae

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genus</th>
<th>G1</th>
<th>G2</th>
<th>N</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Congo</td>
<td>Nairovirus</td>
<td>85–95K</td>
<td></td>
<td>48–60K</td>
<td>Bishop et al. (1980)</td>
</tr>
<tr>
<td>Rift Valley fever</td>
<td>Phlebovirus</td>
<td>65K</td>
<td>56K</td>
<td>25K</td>
<td>Rice et al. (1980)</td>
</tr>
<tr>
<td>Anhembni</td>
<td>Bunyavirus</td>
<td>83–85K</td>
<td>30–33K</td>
<td>20–23K</td>
<td>Obijeski &amp; Murphy (1977)</td>
</tr>
<tr>
<td>La Crosse</td>
<td>Bunyavirus</td>
<td>85K</td>
<td>45K</td>
<td>26K</td>
<td>Obijeski &amp; Murphy (1977)</td>
</tr>
<tr>
<td>Uukuniemi</td>
<td>Uukuvirus</td>
<td>75K</td>
<td>65K</td>
<td>25K</td>
<td>Obijeski &amp; Murphy (1977)</td>
</tr>
<tr>
<td>Hantaan</td>
<td>Ungrouped</td>
<td>72K</td>
<td>56K</td>
<td>45K</td>
<td></td>
</tr>
</tbody>
</table>

The serum used was from a Rattus norvegicus collected during a 1982 serosurvey of rodents in Baltimore (Tsai et al., 1982a). All three antisera precipitated the G1, G2 and N proteins of Hantaan virus. The L protein was not precipitated under these conditions. Normal human serum did not precipitate these viral proteins, nor were they precipitated from uninfected E6 cell extracts reacted with the human and rat antisera.

Comparison with other viruses of the family Bunyaviridae

We compared the electrophoretic profile on SDS–polyacrylamide gels of Hantaan virus proteins with those of several other prototype members of the Bunyaviridae (Fig. 5a, b). Cell extracts were also prepared from these infected cultures and immunoprecipitated with homologous and heterologous sera and MAFs. The results confirmed the serological result, reported by Tsai et al. (1982a), that the anti-Anhembni MAF showed a weak one-way cross-reaction with the nucleocapsid protein in the Hantaan cell extract. None of the anti-Hantaan sera or MAFs immunoprecipitated Anhembni cell extracts. There were no other crossreactions.

The molecular weights of the proteins of four recognized genera of the family Bunyaviridae are different from those of Hantaan virus. The nucleocapsid proteins of La Crosse, Rift Valley fever, Uukuniemi and Anhembni viruses are substantially smaller than that of Hantaan virus. The nucleocapsid protein of Congo virus is closer in size to that of Hantaan than to those of the other three viruses (Table 1).
Fig. 5. Comparison of polypeptides of *Bunyaviridae* on 12.5\% acrylamide gels. (a) Four members of the *Bunyaviridae* were labelled with $^{35}$S, partially purified through a sucrose cushion and electrophoresed on 12.5\% acrylamide gels. Viruses were grown in E6 cells except for La Crosse which was grown in BHK cells. The arrow indicates the location of the 42K cellular protein. Viruses, with their times of harvest, are in the following lanes: (1) Anhemi, 48 h; (2) Congo, 7 days; (3) Hantaan, 7 days; (4) La Crosse, 48 h; (5) Rift Valley fever, 48 h. (b) Viruses labelled with $^{[3]}$H]leucine, purified on a 20 to 70\% sucrose gradient and electrophoresed on 12.5\% acrylamide gels. Viruses were grown in E6 cells, except Uukuniemi which was grown in BHK cells. Viruses, with their times of harvest, are in the following lanes: (1) Congo, 6 days; (2) Hantaan, 6 days; (3) Rift Valley fever, 48 h; (4) Uukuniemi, 6 days.

Studies are underway to compare the proteins of several newly isolated strains of HFRS viruses. Fig. 6 shows the results of SDS–PAGE analysis of SR-11 (Kitamura *et al.*, 1983) and Tchoupatoulas (T. F. Tsai, S. B. Bauer, J. B. McCormick & T. Kurata, unpublished U.S. isolation) compared with Hantaan 76–118. They vary only in a slight mol. wt. difference in the G1 protein.

**DISCUSSION**

The isolation and description of four virion proteins analogous to those of established members of the family *Bunyaviridae* adds new evidence in support of the inclusion of Hantaan virus in this family. We found the smallest of these proteins to be the nucleoprotein, which is consistent with all prototypes of the four genera of the *Bunyaviridae*, where the smallest virion
Hantaan virus proteins

Fig. 6. Comparison of HFRS viruses. Supernatant fluid from one 75 cm² flask of E6 cells per virus was clarified at 2000 r.p.m. for 10 min, then pelleted at 24000 r.p.m. for 1 h through a sucrose cushion. Harvests were made on day 6 of infection, the cultures having been labelled with [35S]methionine on days 4 to 6. SDS-PAGE analysis was on 12.5% acrylamide gels: (a) normal E6 cell supernatant; (b) Hantaan virus; (c) Tchoupatoulas, and (d) SR-11.

proteins are nucleocapsid-associated (Obijeski & Murphy, 1977). A study by Foulke et al. (1981) has described two proteins, 42K and 30K, both smaller than the 45K nucleocapsid protein of Hazara virus as structural proteins. However, studies of viral proteins in cell extracts often demonstrate the presence of actin in the 42K range (Naito & Matsumoto, 1978); a 42K protein is evident in our Fig. 4 and 5(a) and is clearly a cellular protein. In addition, our studies using Congo virus (Matin strain) confirm the work of others (Bishop et al., 1980; Kuismanen et al., 1982; Struthers & Swanepoel, 1982) that p30 is a non-structural protein. Results in our laboratory confirm the report of Bishop et al. (1980) of the presence of a glycoprotein of Congo virus in the range 85K to 95K (Fig. 5a, b).

Additional analogy with established Bunyaviridae was shown by the presence of two glycosylated proteins of intermediate molecular weights and a larger L protein. The fact that the molecular weights of the proteins of Hantaan virus differ from those of members of the
family, supports the suggestion that Hantaan virus may belong to a new genus within the family (Schmaljohn & Dalrymple, 1983). This notion is supported by three other observations: (i) further electron micrograph studies show that Hantaan virus has a unique structure unlike other members of the Bunyaviridae (M. L. Martin et al., unpublished results); (ii) the 3' end sequence of Hantaan virus is different from those of prototype viruses of the four current genera (Schmaljohn & Dalrymple, 1983); and (iii) no serological cross-reactions have yet been found between Hantaan virus and many members of the four current genera (McCormick et al., 1982; Tsai et al., 1982a).

A key to our study was the ability to grow Hantaan virus to high titres. Passage in E6 cells following a harvest from suckling mouse brain increased virus yields from 10- to 100-fold over other culture substrates (Tsai et al., 1982b). In addition, we found that the use of polyethylene glycol or potassium tartrate gradients destroyed much of the virus; instead, we pelleted virus in the J-21 centrifuge and used sucrose gradients.

We did not see significant differences between proteins immunoprecipitated from the infected cell and virion-related proteins. The same proteins were immunoprecipitated both by neutralizing convalescent serum from two patients recovered from haemorrhagic fever with renal syndrome, as well as with serum from a rodent trapped in Baltimore which also had neutralizing antibody to Hantaan virus (Tsai et al., 1982a). The observation that the virion structural proteins are immunoprecipitated by human convalescent sera from patients who acquired HFRS during the Korean War and rodent serum from a rat captured in Baltimore 30 years later suggests a very close relationship between Hantaan-like viruses isolated in the U.S. with those from Korea. Comparisons of the proteins of additional newly isolated strains from different parts of the world, and current studies using antisera raised against specific isolates, will further our understanding of the antigenic diversity of HFRS viruses.

We gratefully acknowledge the helpful suggestions of Dr Helen Lindsey-Regnery and Dr Frederick A. Murphy, and the excellent photography of A. Ray Simons.

REFERENCES


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(Received 21 December 1983)