Association of serine in position 1124 of Hantaan virus glycoprotein with virulence in mice

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Hantaan virus (HV) of the genus Hantavirus causes a fatal disease in suckling mice following intraperitoneal or intracerebral infection. HV cl-1, which was obtained from the 76-118 strain of HV by growth in Vero E6 cells, exhibited high mortality rates in mice whereas mice infected with HV cl-2 survived without any clinical signs. To determine the molecular basis for the marked difference in virulence, we compared the nucleotide sequences of the large (L), medium (M) and small (S) segments of HV cl-1 genome with those of HV cl-2 and found that there was only one predicted amino acid substitution. This amino acid substitution was in position 1124 of the glycoprotein encoded by the M genome segment, in which serine in HV cl-1 was replaced by glycine in HV cl-2. Although there were several nucleotide and amino acid differences between the parental 76-118 strain and HV cl-1, the serine in position 1124 of the glycoprotein was common to the pathogenic parent and the pathogenic mutant. These results suggest that this substitution may be responsible for the virulence of this hantavirus.

Haemorrhagic fever with renal syndrome is caused by viruses in the genus Hantavirus of the family Bunyaviridae. Hantaviruses have been divided into four antigenic groups: Hantaan, Seoul, Puumala and Prosop, identified by neutralization tests (Schmaljohn et al., 1985; Dantas et al., 1987). Currently four more antigenic groups, Belgrade, Thottapalayam, Thailand and Muerto Canyon, have been added (Hjelle et al., 1994; Nichol et al., 1993; Stone, 1993; Wenzel, 1994). The prototype strain of the genus, Hantaan virus, was originally isolated from the Korean striped field mouse (Adopemus agrarius), and is the aetiological agent of Korean haemorrhagic fever, a severe and sometimes fatal human disease (Lee et al., 1978).

Hantaan virus causes a fatal disease in suckling mice after intraperitoneal or intracerebral infection (McKee et al., 1985; Tsai et al., 1982). HV cl-1, a clone originally plaque-purified in Vero E6 cells from Hantaan 76-118 strain, causes high mortality in suckling mice in contrast with another cloned virus, HV cl-2, which causes no clinical symptoms (Tamura et al., 1989). High titres of virus can be isolated from various organs of mice infected with HV cl-1 for extended periods following infection, whereas, after infection with HV cl-2, virus can be isolated only at low titres. Furthermore, HV cl-1 strongly induces cell fusion, whereas the fusion activity of HV cl-2 is minimal. Although both HV clones elicit similar titres of antibodies in mice, cytotoxic T cell activity is induced in mice infected with HV cl-2, but not in mice infected with HV cl-1. To ascertain the molecular basis for such differences in the two clones of Hantaan virus, we have determined the complete nucleotide sequences of the large (L), medium (M) and small (S) segments of both HV clones and found that between the two clones there was only a single amino acid substitution at residue 1124 of the glycoprotein encoded by the M segment. In this paper, we also discuss how one amino acid substitution between HV cl-1 and cl-2 may alter the cell fusion function and the cytotoxic T cell activity which are important in the pathogenesis of Hantaan virus infection.

Vero E6 cells were cultured as monolayers using Eagle's MEM containing 200 mM-glutamine, 10% fetal calf serum (FCS) and 60 mg/l kanamycin. Stock viruses, HV cl-1 and HV cl-2 (Tamura et al., 1989), were prepared in Vero E6 cell cultures, from which the cell debris was removed by centrifugation at 10000 g for 10 min, the supernated being dispensed in small vials and stored at −80 °C.

Confluent monolayers of Vero E6 cells in 75 cm² cell culture flasks were infected with HV cl-1 and HV cl-2 at

The nucleotide sequence data reported in this paper has been deposited with the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the following accession numbers D25528 (HV cl-1, L segment), D25529 (HV cl-1, M segment), D25530 (HV cl-1, S segment), D25531 (HV cl-2, L segment), D25532 (HV cl-2, M segment) and D25533 (HV cl-2, S segment).

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an m.o.i. of 0.1 p.f.u./cell. After adsorption of virus for 1 h at 37 °C, the cells were washed and fed with 20 ml of medium containing 3% FCS. Ten days after infection monolayers were washed once with PBS and harvested. The virus-infected cells were suspended in 4 M-guanidinium isothiocyanate with 0.5% sodium N-lauroyl-sarcosine and 0.1 M-2-mercaptoethanol. Total cellular RNA was extracted by the guanidinium isothiocyanate method (Chirgwin et al., 1979).

Reverse transcriptase–polymerase chain reaction (RT–PCR) and direct sequencing were carried out with suitable synthesized oligonucleotide primers based on the reports by Schmaljohn et al. (1986, 1987, 1990). RT reactions for the cDNA synthesis of the whole HV genome were performed in a 20 μl solution containing 50 mM-Tris–HCl pH 8.3, 50 mM-KCl, 10 mM-MgCl₂ and 3 mM-DTT and were carried out at 42 °C for 30 min with 20 units of RAV2 RT (Takara Shuzo, Kyoto), 5 μg of cellular RNA and 4 μg of random hexamers. PCR was performed with AmpliTaq DNA polymerase (Cetus) using a Thermal Reactor (Taitec, TR-100) for 25 cycles. The reaction mixture was made up of 25 mM-Tris–HCl pH 9.0, 50 mM-KC1, 2 mM-MgCl₂, 1 mM-DTT and 1-25 units of AmpliTaq. The temperature profiles for PCR were: 90 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min. Double-stranded DNA fragments generated by PCR were directly sequenced using the method of Isegawa et al. (1992). The terminal sequences were determined with modified anchored PCR as follows. About 5 μg total RNA was reverse transcribed with specific primers instead of the random hexamers as described above, which were L-2 (5’ TTTATAGGGTTGTGGGATCT CACC 3’) and L-31 (5’ GAAATATAGATAGAACG AGCAT 3’) for the 3’ and 5’ end of the L segment, CM-2 (5’ TCAATGATATTTGACTAGC GT 3’) and CM-19 (5’ AGTATGATATGATGGGCCACC GC 3’) for those of the M segment, CS-2 (5’ TTGTCA ACATATACAGGCTTTC 3’) and CS-7 (5’ ATTA TCTTTTATGATTAC 3’) for those of the S segment, respectively. After incubation, the ssDNA was extracted with phenol-chloroform, mixed with 0.6 volumes of 20% (w/v) of polyethylene glycol 6000 containing 2.5 M-NaCl for 10 min at 37 °C, then centrifuged for 10 min at 15000 g. The pellet was washed with 80% (v/v) ethanol, dried in vacuo, and then dissolved in 20 μl 100 mM-sodium cacodylate containing 2 mM-MnCl₂, 0.1 mM-DTT, 1 mM-dGTP, and 5 units terminal deoxynucleotidyl transferase. The mixture was incubated at 37 °C for 1 h, then at 70 °C for 10 min. After incubation, the mixture was precipitated with ethanol and suspended in 20 μl of 10 mM-Tris–HCl and 1 mM-EDTA (pH 7.5). Portions (1 μl) of the mixture were PCR amplified, using the specific primers described above and anchored primers, which were mixed with OriC (5’ ATA-

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>76-118*</th>
<th>cl-1</th>
<th>cl-2</th>
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<tr>
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<td>C†</td>
<td>T</td>
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<tr>
<td>163</td>
<td>A†</td>
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<tr>
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<td>G†</td>
<td>A</td>
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</tr>
<tr>
<td>1392</td>
<td>G</td>
<td>A</td>
<td>G</td>
</tr>
</tbody>
</table>

* Nucleotide sequence of strain 76-118 was reported by Schmaljohn et al. (1986).
† Nucleotides that resulted in an amino acid change with respect to HV cl-1. Numbers correspond to those of HV cl-1 beginning at the viral-complementary sense RNA.

CATAACCTTATGTATCATACCCCCCCCCCC 3’ and SP6-2 (5’ ATACATAACCTTATGTATCATAC 3’) at a ratio of 1:4. PCR products were purified according to Isegawa et al. (1992), and direct sequencing was carried out with suitable synthesized oligonucleotide primers from positions about 150 bases upstream from the oligodeoxyguanine-tailed ends. In addition, the nucleotide sequences of both ends of each segment were confirmed with the 10 clones after cloning of PCR products in TA cloning vector (Invitrogen).

The S segment of HV genome contains 1696 nucleotides and encodes the nucleocapsid protein (N) in the virus-complementary sense RNA (Schmaljohn et al., 1986). Comparison of the nucleotide sequences of the S segment among HV cl-1, HV cl-2 and the 76-118 virus showed greater than 99% identity (Table 1). The open reading frames (ORFs) in the S segment of HV cl-1 and HV cl-2 had a coding capacity of 430 amino acids, which was one amino acid longer than that of 76-118 virus; synthesis from these ORFs initiated at nucleotides 34 to 36 and terminated at nucleotides 1324 to 1327, respectively. Furthermore, another potential ORF in the same reading frame had a coding capacity of 48 amino acids and initiated at nucleotides 1330 to 1332 and terminated at nucleotides 1474 to 1476. In the case of Hantaviruses, Schmaljohn et al. (1986) have shown that the N protein is the only S segment gene product, although viruses in other genera of the Bunyaviridae encode a nonstructural protein in their S genome segments in addition to the N protein. Translation from the ORF in the HV cl-1 S segment, even with a nucleotide substitution at position 1392, would not result in an additional nonstructural protein, because the amino acid sequence would be unchanged; that sequence would have the same 48 amino acids as HV cl-2 and 76-118. Thus, the N proteins of HV cl-1 and HV cl-2 were identical in terms of their amino acid sequence (Table 1).
The L segment of HV was previously shown to contain 6530 nucleotides and to encode an RNA-dependent RNA polymerase in the virus-complementary sense RNA (Schmaljohn, 1990). The L segments of HV cl-1 and cl-2 were 6533 nucleotides long, equal to the length of corrected sequence of the L segment of the 76-118 strain (GenBank X55901) and they were more than 99% identical (Table 3). The ORFs in the L segment of HV cl-1 and cl-2 had a coding capacity of 2151 amino acids, initiated at nucleotides 38 to 40 and terminated at nucleotides 6491 to 6494. The L segment of 76-118 virus reported by Antic et al. (1992) had a total of eight amino acid changes as compared to that of HV cl-1, at positions 775, 1090, 1587, 1623 and 1624 (Table 2). The two nucleotide differences between HV cl-1 and HV cl-2 found in the ORF at nucleotides 2506 and 5014 did not lead to any change in the amino acids encoded. Thus, the L gene product of HV cl-1 was identical in its amino acid sequence to that of HV cl-2 (Table 2).

The M segment of HV contains 3616 nucleotides and encodes the envelope proteins (G1 and G2) in the virus-complementary sense RNA (Schmaljohn et al., 1987). The nucleotide sequences of the M segments of HV cl-1, HV cl-2 and the 76-118 showed greater than 99% identity (Table 3). The ORFs of HV cl-1 and HV cl-2 M segments had a coding capacity of 1135 amino acids, initiated at nucleotides 41 to 43 and terminated at nucleotides 3446 to 3449; the 76-118 strain has the same capacity and initiates at the same positions. There were two nucleotide differences in the ORF between HV cl-1 and HV cl-2. Although the difference at nucleotide position 46 did not cause an amino acid change, the difference at nucleotide position 3410 altered the serine residue (at amino acid residue 1124 of HV cl-1) to a glycine residue (at 1124 of HV cl-2) (Table 3). Some slight differences exist between the deduced amino acid sequences of the G1 and G2 proteins of HV cl-1 and strain 76-118 at positions 37, 64, 173, 312, 403 and 1044. Significantly, as in strain 76-118, which is also virulent in mice, strain HV cl-1 had a serine in position 1124 of the glycoprotein precursor.

HV cl-1 and cl-2 had previously been shown to differ in their virulence and infectivity for mice, their ability to induce CTLs and cell fusion properties. We demonstrate that the deduced amino acid sequences of the nucleocapsid protein, G1 and G2 proteins and RNA polymerase of this pair of viruses are identical except for a single amino acid substitution in the G2 protein. We propose that the differences in infectivity, CTL induction and cell fusion activity are induced by this substitution. Although the difference in virulence is thought to be caused by the amino acid substitution and/or sequence differences in the terminal non-coding sequences, there were no differences in mortality between HV cl-1- and cl-2-infected SCID mice (Arikawa et al., unpublished data). This result suggests that the nucleotide sequence differences between these two viruses are not directly responsible for the altered virulence but cause an altered immunological response in the host. In other words, a single amino acid substitution in G2 protein may lead to the increase in cytotoxic T cell activity and this increase may be related to the decrease in HV cl-2 pathogenicity.

In La Cross virus, another member of the Bunyaviridae,
the L segment is a major determinant of neurovirulence, whereas the M segment has no influence on neurovirulence [M is important, however, as the major determinant of neuroinvasiveness (Endres et al., 1991)]. The diminution in virulence, caused by the M segment mutation in La Cross virus, may be related to a decrease in viral infectivity for the target organ. The product of the HV M segment serves as a precursor to the G1 and G2 envelope glycoproteins (Schmaljohn et al., 1987). G1 and/or G2 play an important role in the recognition of the host-cell receptor and in the fusion of the cellular and viral membranes. The fusion efficiency of HV cl-2 was lower than that of HV cl-1. In HV cl-2-infected mice, virus titres from various organs are much lower than those in HV cl-1-infected mice, although no difference was found in growth of the two viruses in Vero E6 and peritoneal exudate cells collected from newborn mice (Tamura et al., 1989). Thus, one amino acid substitution in the HV cl-2 (serine to glycine at position 1124 in the glycoprotein) may change infectivity in suckling mice. The difference between HV cl-2 virulence for SCID mice and for normal suckling mice suggests that the decrease in HV cl-2 infectivity in suckling mice is related to the increase in cytotoxic T cell activity rather than to a decrease in fusion efficiency.

We compared the predicted secondary structure of the
HV envelope protein, in the cell-fusion region, with those of other hantaviruses. This site is located near the C terminus of the G2 protein. Most algorithms that predict secondary structure perform poorly when applied to transmembrane domains because they are based on data from proteins in aqueous solution. With this limitation in mind, we subjected the C-terminal transmembrane sequences to three different algorithms which predicted α-helices of variable lengths, but at least 23 residues, from position 1104 to 1126, were indicated to be needed to span the lipid bilayer (Table 4). Based on these predictions we think that position 1124 is located at the border between the transmembrane domain and the cytoplasmic tail. A comparison of the amino acid sequences of the transmembrane and cytoplasmic domains, from residues 1081 to 1135, showed more than 50% identity between the Hantaan, Seoul, Thailand, Puumala, Prospect Hill and Muerto Canyon viruses (Schmaljohn et al., 1987; Isegawa et al., 1990; Arikawa et al., 1990; Xiao et al., 1994; Giebel et al., 1989; Parrington et al., 1991; Spiroupolou et al., 1994) (Fig. 1). The amino acid sequences within each group are more similar to one another than to sequences from other virus strains (e.g., 50% identity between the Hantaan, Seoul, Thailand, Puumala, Prospect Hill and Muerto Canyon viruses but not Puumala viruses). The corresponding amino acids of Puumala viruses are threonine or alanine. The amino acid at this position in the Thailand virus, which causes no clinical signs, is glycine as in HV cl-2. The Prospect Hill virus, which is not known to be associated with human disease, also had a serine residue in the corresponding position (Parrington et al., 1991); its lack of virulence may be due to some factor other than the loss of the serine. These results suggest that there is no correlation between the presence or absence of serine 1124 and the virulence properties of hantaviruses in general.

Palmitoylation influences the fusion activity of influenza virus (Naive & Williams, 1990). The influenza virus haemagglutinin contains three potential acylation sites at cysteine residues in the cytoplasmic domain of the molecule. The modification from cystine to alanine of any single site was sufficient to completely abolish membrane fusion activity (Naive & Williams, 1990). Veit et al. (1991) showed that mutagenesis from cysteine to serine of the acylation sites severely inhibits palmitoylation but that it does not impair haemagglutinin-induced polykaryon formation. In the case of HV it is predicted that two residues, a serine and a cysteine in positions 1124 and 1127 of G2, are palmitoylated, because palmitic acid is linked either to serine/threonine residues via oxyester bonds (Stoffel et al., 1983) or to cysteine residues through thioester linkage (Schmidt, 1989). Our preliminary experiments suggest that the G2 protein of HV is palmitoylated (Isegawa et al., unpublished data). Although we do not yet know whether a serine at position 1124 of the G2 glycoprotein is acylated, we propose that the alteration from serine to glycine is involved in a reduction in fusion activity.

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