Expression of non-conserved regions of the S genome segments of three hantaviruses: evaluation of the expressed polypeptides for diagnosis of haemorrhagic fever with renal syndrome

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Haemorrhagic fever with renal syndrome (HFRS) is a serious and often fatal disease caused by viruses in the Hantavirus genus of the family Bunyaviridae. We expressed the entire coding region of the small (S) genome segments of three serologically distinct hantaviruses as soluble proteins in Escherichia coli and evaluated the expressed nucleocapsid proteins (NPs) as antigens for diagnosis of HFRS. We also prepared novel diagnostic antigens by expressing truncated genes from which we deleted amino acid coding regions that were highly conserved among the three viruses. These antigens were analysed for their potential to detect and differentiate between antisera to various hantaviruses by ELISA. ELISA results obtained with HFRS patient sera or with sera from naturally or experimentally infected animals indicate that homologous antigens and antisera reacted to high titre. The truncated NPs were more specific than the complete NPs in distinguishing between possible aetiological agents of HFRS. Our findings demonstrate that prokaryotic expression of portions of the NPs of specific hantaviruses can be used to generate, readily and efficiently, large quantities of antigen that is both sensitive and specific in diagnostic assays for HFRS.

Introduction

Hantaan (HTN), Seoul (SEO) and Puumala (PUU) viruses are members of the Hantavirus genus of the family Bunyaviridae and, respectively, are aetiological agents of severe, moderate and mild forms of haemorrhagic fever with renal syndrome (HFRS) (Lee et al., 1978, 1982; Yanagihara et al., 1984). HFRS is usually acquired by exposure to aerosolized virus contained in the excrement of rodents (Lee et al., 1981a, b). HTN viruses are typically transmitted by Apodemus mice (Lee et al., 1981b), SEO viruses by rats (Lee et al., 1982) and PUU viruses by Clethrionomys voles (Brummer-Korvenkontio et al., 1980). In areas where these rodents co-mingle, the possibility of infection by more than one hantavirus exists. Although hantaviruses are serologically related, and diagnosis of HFRS can generally be accomplished by using authentic HTN virus antigen, such antigen is sometimes neither sensitive enough to identify distantly related hantaviruses, nor specific enough to differentiate between possible aetiological agents in circulation.

Previously, we reported expression of the small (S) genome segment of HTN virus in eukaryotic cells by baculovirus recombinants, and demonstrated the potential of the expressed nucleocapsid protein (NP) for diagnosis of HFRS (Schmaljohn et al., 1988a; Rossi et al., 1990). We found that the expressed antigen could be used reliably to detect antibodies to HTN and SEO viruses, but was not as sensitive as authentic HTN virus antigen for the detection of antibodies to PUU viruses. However, even authentic HTN virus antigen sometimes failed to detect antibodies to PUU virus (Rossi et al., 1990). Consistent with these findings, sequence analyses of the genomes of a number of hantaviruses demonstrated that the structural proteins of HTN and SEO viruses shared greater amino acid sequence homology than did those of HTN and PUU viruses (Antic et al., 1992).

To improve our diagnostic capabilities for HFRS, we used a prokaryotic expression system to generate soluble NPs of three hantaviruses: HTN virus (strain 76118), SEO virus (strain SR-11) and PUU virus [strain CG 1820 (originally reported as Hällnäs B1); Stohwasser et al., 1990]. These viruses are representative of those in each of the three serologically distinct groups of hantaviruses known to cause HFRS. We also expressed truncated NPs of these viruses from genes we constructed by deleting S segment gene regions, which encode amino acid stretches that are highly conserved among a number of hanta-
viruses. Our studies provide a means for the efficient, safe and cost-effective production of large amounts of antigen for HFRS diagnosis.

Methods

Sera and monoclonal antibodies (MAbs). The origins of serum samples from wild-caught rats and HFRS patients are as described in the legends to Tables 3 and 4. Sera from experimentally infected rats were kindly provided by Dr J. Dalrymple, USAMRIID. These sera were prepared by a single intramuscular injection of five each outbred rats with the following virus isolates: HTN virus strains 76-118 (Lee et al., 1978) and HoJo (HOJO) [isolated from a Korean haemorrhagic fever patient (Schmaljohn et al., 1988)]; SEO virus strains 80-39 (Lee et al., 1982), SR-11 [isolated from a laboratory rat in Japan (Kitamura et al., 1983)] and R22 [isolated from a wild rat in China (Song et al., 1984)]; PUU virus strain Sokkamo [isolated from a Clethrionomys vole in Finland (Schmaljohn et al., 1985)] and CG14445 [isolated from Clethrionomys in the former Soviet Union (Tkachenko et al., 1984)]. The animals were bled 28 days after inoculation and convalescent sera from the five animals in each group were pooled for use in an ELISA. MAbs to the NPs of HTN, SEO and PUU viruses (Ruo et al., 1991) were kindly provided by Dr J. B. McCormick (Centers for Disease Control, Atlanta, Ga., U.S.A.)

Construction of expression plasmids. Molecular cloning of the S genome segments has been reported for HTN virus strain 76-118 (Schmaljohn et al., 1986), SEO virus strain SR-11 (Arikawa et al., 1990) and PUU virus strain CG 1820 (originally reported as HգfNls B1; Stobhasser et al., 1990). To construct plasmids for use in expression of the complete NPs, the entire coding regions of the S genome segments of each virus were amplified by PCR. Forward primers began with an EcoRI site, followed by the sequences complementary to the nucleotides 37 to 57 of HTN virus, and 43 to 60 of SR-11 and PUU viruses with respect to the 5' terminus of virus-complementary sense RNA. Reverse primers also began with an EcoRI site followed by sequences complementary to the nucleotides 1307 to 1326 for HTN virus and 1313 to 1333 for SR-11 and PUU viruses. After digestion with EcoRI, the PCR products were ligated to the EcoRI site of the plasmid FLAG-l (International Biotechnologies). To express the relatively unique regions of the S segments of the three hantaviruses, we used gene splicing by overlap extension to generate truncated genes (Horton et al., 1989). Briefly, the two segments to be joined were amplified by separate PCRs. The last 15 nucleotides from the 5' end of the reverse primer for one segment were made complementary to the first 15 nucleotides from the 3' end of the forward primer of the other segment; thus the PCR products share complementary sequences at the ends to be joined. These two PCR products were mixed as templates in a subsequent PCR which employed a forward primer to the first segment and a reverse primer to the second segment. A recombinant product of these two segments was amplified in the first PCR, nucleotides 37 to 285 of HTN virus and 42 to 285 of SR-11 and PUU viruses and the nucleotides 733 to 936 of all three viruses were amplified separately, then were purified by agarose gel electrophoresis and extraction with Geneclean resin (Bio 101). Approximately 10 ng of each of the PCR-amplified fragments was used as a template for the second PCR.

The junctions of the two gene segments, as well as the junctions with the plasmid FLAG-l, were verified by nucleotide sequence analysis with dideoxynucleotide chain termination reactions using double-stranded cDNA templates and Sequenase (United States Biochemical) as previously described (Arikawa et al., 1990). Competent HB101 cells (Gibco) were transformed with each plasmid.

Serological assays. Plaque reduction neutralization tests (PRNTs) were performed as previously described (Schmaljohn et al., 1985). ELISAs with sera from rats and humans or with MAbs were performed as described elsewhere (Schmaljohn et al., 1988a) with the following modifications. Each well of the ELISA plates (Costar, high binding, cat. no. 3590) was coated with 100 ng of recombinant NP in 100 µl of 0.1 M-bicarbonate buffer pH 9.4, and incubated overnight at 4 °C. All reagents were diluted in PBS containing 1 % bovine serum albumin and 0.05% Tween-20. Well contents were aspirated and washed by using an automated ELISA washer (Bio-Rad) and the results were read as absorbance at 405 nm (A405) in an ELISA reader (Bio-Tek Instruments). The ELISA titres were expressed as the reciprocal of the highest dilution of antisera or MAbs resulting in an absorbance reading ≥ the mean of the normal human or rat serum or ascitic fluid plus 3 s.d.

PCR. Reactions were carried out in an automated thermal cycler (Perkin-Elmer Cetus) for 30 cycles. Each cycle consisted of 1 min at 92 °C, 2 min at 37 °C and 3 min at 72 °C, followed by a 10 min incubation at 72 °C. Each primer (100 pmol), 10 ng plasmid template and 1 unit of Taq polymerase (Perkin-Elmer Cetus) were used for each reaction in a total volume of 100 µl containing 50 mM-KCl, 10 mM-Tris-HCl pH 8.3, 1.5 mM-MgCl2 and 0.1 % gelatin.

Expression and purification of gene products. Bacterial growth conditions were as described by the manufacturer for the FLAG-1 system (International Biotechnologies). We extracted the hantavirus NP–FLAG fusion proteins from Escherichia coli after disrupting cells by treating them with lysozyme (final concentration of 0.25 mg/ml) and by repeated freezing and thawing followed by homogenization with a Dounce homogenizer. Affinity purification of the expressed fusion protein with the anti-FLAG MAb was as described by the manufacturer. Binding was in the presence of 1 µM-CaCl2 and protein was eluted with 1 mM-EDTA. The expressed protein was quantified by using a Bio-Rad DC protein assay kit. The complete and truncated NPs of the hantaviruses were cleaved from the FLAG peptide by incubation with enterokinase (1 µg fusion protein/1 unit enterokinase) at 37 °C for 18 h.

Results

Construction of truncated S segment genes

Overall similarities of the deduced amino acid sequences of the NPs of HTN versus SEO, HTN versus PUU and PUU versus SEO viruses are approximately 82 %, 62 % and 62 %, respectively (Antic et al., 1992). Dot matrix comparisons of these amino acid sequences, however, revealed two regions that were relatively unique to each virus (Fig. 1). The first region was located at the amino terminus of each NP and the other in the carboxyterminal half of the protein. We used PCR splicing by overlap extension (Horton et al., 1989) to generate truncated genes containing only these two regions of the NPs. For HTN virus, the truncated gene encoded amino acids 1 to 83 and 233 to 304, with respect to the amino terminus of the NP (Fig. 2). For SEO and PUU viruses, the genes contained coding information for amino acids 1 to 81 and 233 to 304. The resulting truncated NPs displayed amino acid sequence identities of 65 %, 26 % and 30 %, respectively, for HTN versus SEO, HTN versus PUU and PUU versus SEO viruses.
Expression and purification of hantavirus NPs

A prokaryotic expression system (E. coli, FLAG system, International Biotechnologies) was used to express genes representing the entire NP coding regions as well as the truncated genes of each virus. The polypeptides were expressed as fusion proteins with an eight amino acid marker peptide (FLAG peptide). The additional 1K hydrophilic peptide contains an enterokinase cleavage site; thus, after affinity purification of the fusion protein with a MAb specific for the 8 amino acid FLAG peptide, treatment with enterokinase can be used to remove these extraneous amino acids from the purified NP.

Expression levels of the purified NP–FLAG fusion proteins were estimated to be 0.5 to 1.0 mg/l of bacterial culture. Both the full-length and the truncated NPs
remained soluble in aqueous solutions. Examination of
the expressed, purified proteins by PAGE and Coomassie
blue staining revealed polypeptides of the expected
apparent $M_r$ of 48K and 17K for the complete and
truncated NPs, respectively (Fig. 3). A band approxi-
mately twice the $M_r$ of the truncated NP (approximately
34K) was observed routinely and may represent a
multimeric form of the truncated protein. Western blot
analysis with polyclonal antisera to HTN, SEO or PUU
viruses confirmed the authenticity of these polypeptides
(Fig. 4).

**Antigenic assessment of the expressed NPs**

The expressed NPs of the hantaviruses were examined
for their reactivities with MAbs to the actual NPs of
HTN, R22 and PUU viruses by ELISA (Table 1). The
majority of the epitopes recognized by the MAbs
examined were no longer present in any of the truncated
proteins. However, the HTN MAb, HD01, the R22
MAb, JD01, and the PUU MAb, GB04, each retained
reactivity with their homologous truncated NP but did
not recognize heterologous truncated NPs. The R22
MAb, EC01, reacted with the truncated NPs of both
HTN and SEO viruses. In contrast, none of the MAbs
tested displayed specificity for any of the three complete
NPs, although HTN MAbs, GD04, GD05 and KD01,
R22 MAbs, CD03, DC03, JD01 and IC06, and the PUU
MAb, GB04, reacted to higher titre with homologous
antigens than to heterologous antigens (Table 1). These
data indicate that many, but not all, cross-reactive
epitopes detectable by ELISA were removed from the
truncated NPs and that each truncated NP retained at
Fig. 4. Western blot analysis of the *E. coli*-expressed, truncated NPs of HTN, SEO and PUU viruses. Blots were performed using polyclonal rabbit antisera as indicated, and with a non-radioactive detection procedure described in Methods.

Table 1. *ELISA reactivities of *E. coli*-expressed, complete or truncated NPs with hantavirus-specific MAbs*

<table>
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<th>Truncated NP</th>
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<td></td>
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<td>SEO</td>
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<tr>
<td>GB04</td>
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*ELISA titres are expressed as the reciprocal of the highest dilution of antibody resulting in an A450 ≥ mean of the normal ascitic fluid plus 3 s.d.

least one specific epitope. These data were confirmed by Western blot analysis of the truncated proteins by using polyclonal, hyperimmune rabbit sera to HTN (strain 76-118), SEO (strain SR-11) or PUU (strain Sotkamo) (Fig. 3). In this assay, we observed reactivity with each of the three homologous antisera and antigens (although reactivity to PUU antigen was poor even in the homologous system), and the reactivities of the HTN
Table 2. ELISA reactivities of E. coli-expressed, complete or truncated NPs with sera from experimentally infected rats

<table>
<thead>
<tr>
<th>E. coli-expressed antigen</th>
<th>Complete NP</th>
<th>Truncated NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>HTN</td>
<td>SEO</td>
</tr>
<tr>
<td>Hantaan</td>
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</tr>
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<td>76-118</td>
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<tr>
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<tr>
<td>Seoul</td>
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<td></td>
</tr>
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<td>SR-11</td>
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<tr>
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<td>320</td>
</tr>
<tr>
<td>R22</td>
<td>80</td>
<td>80</td>
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<tr>
<td>Puumala</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sotkamo</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>CG14445</td>
<td>10</td>
<td>20</td>
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</table>

* ELISA titres are expressed as the reciprocal of the highest dilution of antibody resulting in an A405 > mean of the normal serum plus 3 s.d.

Diagnostic potential of the expressed NPs

To investigate the potential of the complete and truncated proteins as diagnostic antigens for HFRS, we screened by ELISA a panel of sera from rats experimentally infected with various hantaviruses. Sera from these animals reacted with both the complete and truncated, homologous, expressed NPs (Table 2). More specific reactivities were observed with the truncated NPs. The truncated PUU antigen did not react with antisera to either HTN or SEO viruses but did react with antisera to PUU virus. In contrast, we observed a greater cross-reactivity with truncated SEO and HTN antigens and antisera to HTN or SEO viruses. Nevertheless, for all sera tested there were at least twofold greater reactivities of homologous antigens and antisera than of heterologous antigens and antisera (Table 2).

Currently, the most specific test known for differentiation among hantaviruses is the PRNT (Schmaljohn et al., 1985). We compared the specificity of PRNT to that of ELISA with the expressed, truncated NPs and a panel of antisera obtained from rats naturally infected with hantaviruses, and with a panel of sera from HFRS

Table 3. ELISA reactivities of E. coli-expressed, truncated NPs with sera from naturally infected rats as compared to PRNT of authentic hantaviruses

<table>
<thead>
<tr>
<th>ELISA*</th>
<th>PRNT†</th>
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<tr>
<td></td>
<td>Serogroup</td>
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<td>Serum‡</td>
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* ELISA titres were expressed as the reciprocal of the highest dilution of antisera resulting in an A405 > mean of the normal serum plus 3 s.d.
† PRNT titres are expressed as the reciprocal of the highest dilution neutralizing 50% of 75 to 100 p.f.u.
‡ Serum samples 1 to 10 were from rats trapped in Philadelphia, Pa., U.S.A., samples 11 to 12 from rats trapped in Houston, Tx., U.S.A. and samples 13 to 21 from rats trapped in Belgium (LeDuc et al., 1984).
§ NT, Not tested.
patients. Of the 21 rat sera examined, PRNT identified two infections with HTN virus and 16 infections with SEO virus. Three of the sera (Table 3, sera 1, 4 and 20) did not have a detectable plaque-reduction neutralization response to any of the hantaviruses. Of these three sera, two of them also had no ELISA reactivity to any of the antigens, and the third reacted with the expressed, truncated PUU antisera by ELISA, suggesting an infection with PUU virus (Table 3, serum 4). Of the two infections with HTN virus and 16 infections with SEO virus, 12 were identified as SEO virus both by PRNT and by ELISA, and one (Table 3, serum 9) was identified as a SEO virus by PRNT but had the same titre to HTN and SEO by ELISA. The remaining three sera (Table 3, sera 18, 19 and 21) reacted to higher PRNT titres with SEO virus than with HTN virus but displayed higher ELISA titres with HTN virus antigen than with SEO virus antigen.

Of the 35 sera from HFRS patients, 15 were identified as HTN-specific and 11 as PUU-specific, both by PRNT and ELISA, with the expressed truncated NPs (Table 4). Six samples had no detectable antibodies to any of the three hantaviruses either by PRNT or by ELISA, and one sample had a very low reactivity to the HTN antigen by ELISA (Table 4, sample 12) but did not have a PRNT titre to any of the three hantaviruses. Of the remaining two samples, one had a higher PRNT titre to HTN virus than to PUU virus but reacted with the PUU antigen in ELISA more strongly than to the HTN antigen (Table 4, sample 29). The other serum was identified as containing antibodies to a HTN virus by PRNT but had the same ELISA titre to both HTN and SEO truncated, expressed antigens (Table 4, sample 24).

Discussion

The goal of this study was to develop a safe, simple and cost-effective source of diagnostic antigen for HFRS, which was sensitive enough to detect a wide variety of hantaviruses and specific enough to differentiate among serologically related viruses. Bacterial expression meets the first three criteria: safety, simplicity and cost. Unlike authentic hantavirus antigens, which require containment laboratory conditions for virus propagation, and which are time-consuming and costly to prepare, E. coli-expressed antigens can be generated rapidly, with minimal containment, and scale-up should be readily attainable. We used the FLAG-I-E. coli system to express hantaviral NPs as fusion proteins with an 8 amino acid peptide (FLAG peptide) and an Omp A signal sequence which dictates secretion into the bacterial periplasm. The resultant proteins were purified from lysed bacteria by affinity chromatography with antibodies to the FLAG peptide and, after purification, were treated with enterokinase to remove the extraneous, non-hantaviral portions of the fusion protein. Although our studies were performed with purified, enterokinase-treated antigen, we have also obtained satisfactory results with uncleaved antigen. Thus, for routine use, the expense of enterokinase cleavage may not be necessary.

All of the proteins produced in our studies remained soluble in aqueous solutions. These results differ from those obtained by baculovirus expression of HTN NP (Schmaljohn et al., 1988a) and E. coli expression of complete or terminally truncated HTN and PUU NPs (Gott et al., 1991). In both of those studies, the expressed NPs formed insoluble aggregates in the cytoplasm of host cells, and purification required detergent treatment or denaturing conditions. In contrast, our expressed proteins could be purified in a single step, under mild, non-denaturing conditions.

To improve the sensitivity of the antigens, we prepared NPs by using genes representative of all three serological groups of hantaviruses known to cause HFRS. To improve the specificity of the antigens, we constructed truncated genes that contained coding information for two relatively unique regions of the NPs. The resultant expressed polypeptides were approximately one-third the size of the authentic viral NP.

With MAbs, we demonstrated that each truncated NP retained at least one epitope of the homologous hantavirus, and had many cross-reactive epitopes deleted. The truncated NPs displayed more antigenic specificity than did the complete NPs when reacted with the same antisera. The truncated antigens could be used to differentiate anti-HTN and anti-SEO clearly from anti-PUU antibodies, without one-way cross-reactivity, as found in previous studies (Lee et al., 1985; Sheshberadaran et al., 1988; Zoller et al., 1989; Gott et al., 1991). This result was expected, because the number of identical amino acids in the truncated compared to the complete NP was reduced from greater than 60% to approximately 30%. Also, as expected, because the overall homology of the HTN versus SEO antigen was still 65% in the truncated NPs (as opposed to 82% in the complete NPs), it was more difficult to distinguish anti-HTN from anti-SEO antibodies. Nevertheless, in many cases, we found at least a twofold difference in ELISA titre between the anti-HTN and anti-SEO sera.

One of the most sensitive tests available for serological differentiation among hantaviruses is the PRNT (Schmaljohn et al., 1985). PRNT, of course, measures antibody reactivity with the viral envelope glycoproteins, G1 and G2, both of which have neutralizing determinants (Arikawa et al., 1990). In contrast, our ELISA is based only on antibodies reactive with the NPs of these viruses. The viral NPs are less subject to humoral immune response selective pressures than are the envelope
### Table 4. Differentiation of human hantavirus infections by ELISA versus PRNT

<table>
<thead>
<tr>
<th>E. coli-expressed antigen</th>
<th>Virus</th>
<th>Serum†</th>
<th>HTN</th>
<th>SEO</th>
<th>PUU</th>
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* ELISA titres were expressed as the reciprocal of the highest dilution of antisera resulting in an A405 ≥ mean of the negative control serum plus 3 S.D.  
† PRNT titres are expressed as the reciprocal of the highest dilution neutralizing 50% of 100 p.f.u. of virus.  
‡ Serum samples were obtained from HFRS patients residing in geographic regions listed.  
§ NT, Not tested.  
||, Unable to determine.

This is reflected in the finding that the NPs of hantaviruses share slightly higher amino acid identities than do the envelope glycoproteins (Antic et al., 1992). Thus, it is reasonable to assume that a diagnostic assay based upon the viral NPs may be more likely to succeed in identifying distantly related hantaviruses, than would an assay based on antibody reactivity to the envelope glycoproteins. We compared our truncated NP ELISA with PRNT by using sera from wild-caught rats, or from HFRS patients. With the rat sera, the ELISA and PRNT differed in identifying five of 21 sera. Three of the sera were from rats trapped in Belgium, and were identified as anti-HTN by ELISA and anti-SEO by PRNT. The ELISA titres were all at least fourfold higher for HTN than for SEO, and one of the three was eightfold higher by ELISA, but displayed only a twofold higher titre to
had a titre of 20 by ELISA to both HTN and SEO, and hantaviruses, either in the laboratory or in nature, it is possible that infection of a rodent with more than one reactive virus, different to any of the three prototypes, infected this rat. Without a virus isolation, it is impossible for us to determine whether the ELISA reactivity of this rat serum indicates a natural infection, or whether the results indicate a false positive.

Although there have been no reports of gene segment reassortment occurring among serologically distinct hantaviruses, either in the laboratory or in nature, it is possible that infection of a rodent with more than one hantavirus could lead to reassortant viruses. Such viruses could not be accurately diagnosed by ELISA with our NP antigen. The good correlation between our ELISA and PRNT (an assay dependent on M segment gene products) would suggest that reassortants are not a major consideration with the samples we tested. Nevertheless, reassortant viruses should not be discounted when diagnostic results are unclear.

Like the rat sera, results with sera from HFRS patients were the same by PRNT and by ELISA in most cases (three differences out of 35 examined). One of the three differences occurred among negative control sera, in that an ELISA titre of 20 was found to PUU. Another serum, from Korea, displayed equivalent ELISA titres to SEO and HTN, but had an at least fourfold higher PRNT titre to HTN than to SEO. The final differing result was with a German patient’s serum sample. This serum had a fourfold higher ELISA titre to PUU than to HTN, and a fourfold higher PRNT titre to HTN than to PUU. In this case, it seems reasonable that the ELISA was correct in identifying the infecting agent as PUU, in that all of the other samples from Germany were identified as originating from PUU infections both by PRNT and ELISA.

In conclusion, we have developed a means of generating quantities of antigen for diagnosing HFRS with a bacterial system. Results obtained by ELISA using this antigen compared favourably with results obtained by PRNT. Although exquisitely sensitive, PRNT is not a practical diagnostic tool for hantavirus infections, in that assays take from 1 to 3 weeks, are extremely difficult to perform and are not even possible for all hantaviruses. In contrast, ELISA is a simple, established method for diagnosis and is routinely used in field laboratories. The antigen described here is easy to prepare and can be adapted for an ELISA designed to detect a wide variety of hantaviruses (i.e. with a mixture of the truncated or complete proteins) or to identify serologically distinct viruses (i.e. with individual truncated proteins).

References


SCHMALJOHN, C. S., SUGIYAMA, K., SCHMALJOHN, A. L. & BISHOP,


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