VIROLOGY 216, 397–406 (1996)
ARTICLE NO. 0075

Characterization of Puumala Virus Nucleocapsid Protein: Identification of B-Cell Epitopes

ÅKE LUNDKVIST,*,†,[1] HANNIMARI KALLIO-KOKKO,‡ KATARINA BRUS SJÖLANDER,* HILKKA LANKINEN,‡
BO NIKLASSON,*,§ ANTTI VAHERI,‡ and OLLI VAPALAHTI‡

*Department of Defense Microbiology, Swedish Institute for Infectious Disease Control, S-105 21, Stockholm, Sweden; and †Department of Virology, Karolinska Institute, S-171 77, Stockholm, Sweden; §National Defense Research Establishment, FOA-4, S-901 82, Umeå, Sweden; and ‡Department of Virology, Haartman Institute, University of Helsinki, P.O. Box 21, FIN-00014 Finland

Received September 27, 1995; accepted December 14, 1995

B-cell epitopes in the nucleocapsid protein (N) of Puumala (PUU) virus were investigated by use of truncated recombinant proteins and overlapping peptides. Six of seven epitopes, recognized by bank vole monoclonal antibodies, were localized within the amino-terminal region of the protein (aa 1–79). Polyclonal antibodies from wild-trapped or experimentally infected bank voles identified epitopes located over the entire protein. Antibody end-point titers to different N fragments indicated that the amino-terminal region is the major antigenic target in PUU virus-infected bank voles. To investigate the role of PUU virus N in protective immunity, we analyzed the immunogenicity of truncated recombinant N and developed an animal model based on colonized bank voles. No PUU virus N antigen, nor any glycoprotein-specific antibodies, could be detected after virus challenge in animals immunized with an amino-terminal fragment (aa 1–118), a fragment covering two thirds of the protein (aa 1–267), or total N, indicating that a complete protection was evoked by the recombinant proteins. Two of eight animals immunized with shorter N fragments displayed either N antigen, or glycoprotein-specific antibodies, suggestive of partial protection. Prechallenge sera from all groups of immunized animals were found negative or only weakly positive for neutralizing antibodies when assayed by focus reduction neutralization test, which indicated an important role for cell-mediated immunity in protection.

INTRODUCTION

Hantaviruses, members of the family *Bunyaviridae,* are enveloped negative-stranded RNA viruses with tripartite genomes (Schmaljohn *et al.,* 1985). The *Hantavirus* genus is comprised of at least eight serologically and genetically distinct groups of viruses: Hantaan (HTN), Seoul (SEO), Puumala (PUU), Prospect Hill (PH), Dobrava, Thailand, Thottapalayam, and Sin Nombre/Four Corners viruses (Schmaljohn *et al.,* 1985; Schmaljohn *et al.,* 1995; Nichol *et al.,* 1993; Chu *et al.,* 1994; Hjelle *et al.,* 1994a; Xiao *et al.,* 1994). In addition, nucleotide sequence and immunological data on five potentially new serotype viruses, Tula (TUL) (Plyusnin *et al.,* 1994), El Moro Canyon (Hjelle *et al.,* 1994b), Khabarovsk (Dzagurova *et al.,* 1995; Hörling *et al.,* unpublished observation), Bayou (Morzunov *et al.,* 1995), and Black Creek Canal (Rollin *et al.,* 1995) have been reported. Small mammals, mainly rodents, are the natural reservoirs of hantaviruses and transmission to humans occurs via aerosolized animal excreta (Elliot, 1990).

HTN, SEO, and PUU viruses, carried by the striped field mouse (*Apodemus agrarius*), rats (*Rattus norvegicus* and *R. rattus*) and the bank vole (*Clethrionomys glareolus*), respectively, cause clinically similar human diseases, referred to as hemorrhagic fever with renal syndrome (HFRS). The diseases are characterized by fever, renal failure, and, in severe cases, hemorrhagic manifestations (Yanagihara and Gajdusek, 1988). The clinical manifestations of HFRS are generally most severe for infections caused by HTN virus, less severe for SEO virus, and milder for PUU virus. The mortality of HFRS varies between 0.2 and 10% and approximately 150,000 cases occur annually world wide (for review see Lundkvist and Niklasson, 1994). In 1993, a new hantavirus-caused human disease, called hantavirus pulmonary syndrome (HPS), was discovered in the United States (Nichol *et al.,* 1993). HPS, characterized by acute respiratory distress, has a mortality of approximately 50%. The etiologic agent of HPS, designated Sin Nombre/Four Corner viruses, are carried primarily by the deer mouse (*Peromyscus maniculatus*) (Nichol *et al.,* 1993; Hjelle *et al.,* 1994a; Spiropoulou *et al.,* 1994).

The genomes of hantaviruses encode four structural proteins: the L-segment encodes a virus-associated RNA-dependent RNA polymerase, the M-segment two glycosylated envelope proteins (G1 and G2), and the S-segment a nucleocapsid protein (N) (Schmaljohn and Dalrymple, 1983; Antic *et al.,* 1991). The envelope glyco-

---

[1] To whom correspondence and reprint requests should be addressed at Department of Defense Microbiology, Swedish Institute for Infectious Disease Control, S-105 21, Stockholm, Sweden. Fax: +46-8-7303248. E-mail: Ake.Lundkvist@smi.ki.se.

0042-6822/96 $18.00

proteins are presumed to be the major elements involved in induction of immunity to hantaviruses, since monoclonal antibodies (Mabs) to G1 and G2, but not to the nucleocapsid protein (N), have been found to neutralize viral infectivity *in vitro* (Dantas *et al.,* 1986; Arikawa *et al.,* 1989; Lundkvist and Niklasson 1992). The importance of the humoral response for protection against HTN virus infection has been demonstrated by passive transfer of immune sera or Mabs and subsequent challenge with virus (Zhang *et al.,* 1989; Schmaljohn *et al.,* 1990; Arikawa *et al.,* 1992). A cell-mediated immune response to HTN virus has also been implicated in protection (Asada *et al.,* 1988, 1989; Yoshimatsu *et al.,* 1993). The actual importance and protein specificity of the cellular response has, however, not been resolved. Recombinant HTN virus glycoproteins, expressed by baculovirus and vaccinia virus vectors, have been shown to protect hamsters from infection. For complete protection, recombinants expressing both G1 and G2 were needed (Schmaljohn *et al.,* 1990). Passive transfer of spleen cells from mice immunized with recombinant G1/G2 partially protected suckling mice from infection (Yoshimatsu *et al.,* 1993). Interestingly, HTN virus recombinant N (rN) has been shown to protect hamsters and suckling mice from HTN virus infection (Schmaljohn *et al.,* 1990; Yoshimatsu *et al.,* 1993). Moreover, a Mab specific for HTN N has been shown to protect mice from infection (Yoshimatsu *et al.,* 1993).

We have recently identified B-cell epitopes on the PUU virus N recognized by the polyclonal antibody response in humans (Lundkvist *et al.,* 1995a; Vapalahti *et al.,* 1995). With a panel of PUU virus bank vole Mabs, reactive with eight distinct epitopes on N, only one epitope could be defined when overlapping 10-mer amino acid peptides were used (Lundkvist *et al.,* 1995a).

To further define the localization of B-cell epitopes recognized by the natural reservoir of PUU virus, the bank vole, we chose to use longer synthetic peptides and truncated rN proteins. To investigate the role of PUU virus N in protective immunity, we analyzed the immunogenicity of truncated rN proteins and developed an animal model. Our results show that several B-cell epitopes are located in the amino-terminal part of N, and that N produced in insect cells and as GST-fusion proteins in *Escherichia coli* elicit strong antibody responses and protect animals from infection with PUU virus.

MATERIALS AND METHODS

Virus strains

PUU virus prototype strain Sotkamo (Brummer-Korvenkontio *et al.,* 1980; Vapalahti *et al.,* 1992) was propagated in Vero E6 cells (CRL 1586, ATCC) cultivated in Eagle's MEM supplemented with 2% fetal calf serum (FCS), 2 m*M* L-glutamine and antibiotics. PUU virus strain Kazan was passaged in colonized bank voles as previously described (Gavrilovskaya *et al.,* 1983).

Antibodies

Generation and characterization of PUU virus N-, G1-, and G2-specific bank vole Mabs have been described elsewhere (Lundkvist *et al.,* 1991; Lundkvist and Niklasson, 1992). Briefly, colonized bank voles were infected with PUU virus obtained from lung tissues of bank voles trapped in northern Sweden. [Bank vole × mouse] heterohybridomas were established and PUU virus-specific clones were selected after screening against native virus.

Large scale Mab production was performed by culturing hybridomas in roller bottles followed by purification on protein-G Sepharose as previously described (Lundkvist *et al.,* 1991). Polyclonal antisera to Keyhole limpet hemocyanin (KLH)-conjugated peptides (Berzins *et al.,* 1986) were raised in bank voles and in Balb/c mice by three biweekly, subcutaneous (sc) immunizations of 100 μg peptide-conjugate emulsified in Freund's complete adjuvant, Freund's incomplete adjuvant, and phosphate-buffered saline (PBS), respectively.

Peptide P4

The peptide P4, a 30 amino acid (aa) carboxy-terminal amide corresponding to residues 241–270 (EKECPFIKPEVKPGTPAQEIEMLKRNKIYF) of PUU virus N, was synthesized by Scandinavian Peptide Synthesis (Köping, Sweden).

Recombinant PUU virus N proteins

The production of PUU N in insect cells with a recombinant baculovirus (bac-PUU-N) has been described in detail elsewhere (Vapalahti *et al.,* 1996). Briefly, the antigen consisted of an extract of *Spodoptera frugiperda* (Sf9) cells containing approximately 35% of bac-PUU-N, solubilized with urea, and passed through Sephadex columns into an aqueous buffer with proteinase inhibitors. The areas coding for truncated N proteins, expressed as GST-fusion proteins in *E. coli,* were individually amplified by PCR using the PUU virus Sotkamo strain S cDNA (Vapalahti *et al.,* 1992) as a template, cut with restriction enzymes and cloned to the *Bam*HI and *Eco*RI sites of pGEX2T vector (Pharmacia, Uppsala, Sweden) as follows: Construct rN 1a coding for aa 1–79 with primers A: (TTTGAATTCTCTAGATCTGGAATGAGTGACTTGACAGA) and B: (TTTGAATTCTTGGATCCAGTCGGTTAAGTAGGTTTAGTA), construct rN 1b coding for aa 1–118 with primers A and C: (TTTGAATTCGTCGACTCAATCTGCTGTTTGGCCACTTG), construct rN 3 coding for aa 229–327 and six extra aa from the polylinker area with primers D: (TTTGTCGACGGATCCAAGGATTGGTCTGAGAGAA) and E: (TTTGAATTCGTCGACTCAGCAACATAGATACATGTTGG), construct rN 2b coding for aa 135–

214 with primers F: (TTTGTCGACGGATCCAAAGCTTTATACATGTCTC and G: (TTTGAATTCGTCGACTCAGTTAGCAACCTGGATCTGAG), construct rN 2c coding for aa 135–327 and six extra aa from the vector with primers F and E. Extra stop codons and the natural initiation codon of PUU N are underlined, restriction sites used for cloning are shown in bold. The cloning and expression of constructs rN 2/3 (aa 1–267, GST-PUU-N2/3, Vapalahti *et al.,* 1995) and rN Tot (total coding area, GST-TUL-N, Plyusnin *et al.,* 1994) and rN Eco (aa 1–61, GST-TUL-N-delta) have been described previously. The plasmid pGEX2T without an insert coding only for the GST was used as a control (GST-c).

The expression of the GST-fusion proteins was performed as recommended by the manufacturer (Pharmacia). Briefly, overnight cultures of transformed BL21 bacteria (Studier *et al.,* 1990) were diluted 1/100 and grown for 2 hr at 37°, induced with 0.5 m*M* IPTG for 3 hr after which the cells were pelleted. GST-fusion proteins were purified by binding to Glutathione Sepharose 4B beads (Pharmacia) in the presence of 2% *N*-lauroylsarcosine (Sigma, St. Louis, MO) as described by Frangioni and Neel (1993). The protein was eluted from the beads by 25 m*M* reduced glutathione (Boehringer Mannheim, Mannheim, Germany) and dialyzed.

Immunoblotting

Bacterial cell suspensions were suspended in Laemmli's sample buffer and proteins were separated in SDS–PAGE and transferred to Immobilon filters (Millipore, Bedford, MA). After blocking, filters were subsequently incubated with Mabs (1 μg/ml) overnight at 4°, followed by rabbit anti-mouse peroxidase-conjugate (Dakopatts, Denmark), diluted 1:400, for 1 hr at 20°. Blots were visualized by addition of 1-1-diaminobenzidine substrate.

Epitope mapping (PEPSCAN)

The PEPSCAN method (Geysen *et al.,* 1987), designed for identification of linear B-cell epitopes, was used to locate antibody-reactive peptides comprised within the sequence of N of PUU virus prototype strain Sotkamo. In total, 141 peptides (14-mer overlapping peptides covering the complete N by a shift of three amino acids; Vapalahti *et al.,* 1995) were examined. Antibody reactivities with PEPSCAN peptides were measured by ELISA as previously described (Geysen *et al.,* 1987), using polyclonal bank vole sera (diluted 1:200). Bound antibodies were detected with alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG and *p*-nitrophenyl phosphate substrate according to the manufacturer's instructions (Sigma).

PUU virus rN ELISA

Antibody reactivities of wild-trapped and experimentally infected bank voles to PUU rN were examined by ELISA. PUU rN fragments, 5 μg/ml in coating buffer (0.05 *M* carbonate buffer, pH 9.6) of purified proteins or 1:200 dilutions of nonpurified proteins, or nonpurified bac-PUU-N (diluted 1:1000), were adsorbed to microtiter plates overnight at 4°. Unsaturated protein-binding sites were blocked by addition of 3% bovine serum albumine (BSA) in PBS for 1 hr. Serial dilutions of sera in ELISA buffer (PBS with 0.5% BSA and 0.05% Tween-20) were preincubated with 1% GST-c for 1 hr before addition to plates. Adsorbed sera were incubated for 1 hr followed by ALP-conjugated goat anti-mouse IgG and *p*-nitrophenyl phosphatase substrate according to the manufacturer's instructions (Sigma). All incubations (100 μl/well) were performed at 37° and plates were washed five times in washing buffer (0.9% NaCl and 0.05% Tween 20) between each step. End-point titers were expressed as the reciprocal of the maximum dilution of sera giving a ratio of >3 times that obtained with control antigen.

To investigate antibody reactivity to the carboxy-terminal of PUU virus N (aa 328–433), bank vole immune sera were preabsorbed to rN 2/3 and rN 3 (together corresponding to aa 1–327) before testing for reactivity to bac-PUU-N (covering total N). Sera (diluted 1:200) were incubated with a cocktail of rN 2/3 and rN 3 (20 μg/ml of each protein) for 2 hr at 20°.

PUU virus G1/G2 ELISA

Bank vole sera were analyzed for presence of antibodies reactive with the PUU virus envelope glycoproteins (G1 and G2) by an ELISA. The human Mabs G1-1E7-1E5 and 1C9 (Lundkvist *et al.,* 1993a; 1993b), specific for PUU virus G1 and G2 (epitopes G1-b and G2-a2), respectively, 5 μg/ml in coating buffer, were adsorbed to microtiter plates overnight at 4°. All the following incubations were performed for 1 hr at 37° with five washes between each step. Unsaturated protein binding sites were blocked with 3% BSA in PBS. Detergent-treated native PUU virus antigen prepared as previously described (Lundkvist and Niklasson, 1992) or control antigen (diluent only), followed by serial twofold dilutions of sera (starting at 1:200), were added to the plates. ALP-labeled donkey anti-mouse IgG (Jackson, West Grove, PA), followed by *p*-nitrophenyl phosphate substrate (Sigma), were used to detect specific antibody binding according to the manufacturer's instructions. End-point titers were determined as described above.

The specificity of the assay was examined by Mab reactivity. Bank vole Mabs 5A2 and 5B7, specific for additional G1- and G2-epitopes (G1-a and G2-b), respectively, and consequently not interactive in the antigen binding by the human Mabs used for antigen capture, and the N-specific Mab 3E11, were conjugated to biotin as described elsewhere (Harlow and Lane, 1988). Biotinylated Mabs (0.1 μg/ml) were incubated for 1 hr. Peroxidase-conjugated streptavidin (Sigma), followed by tetrameth-

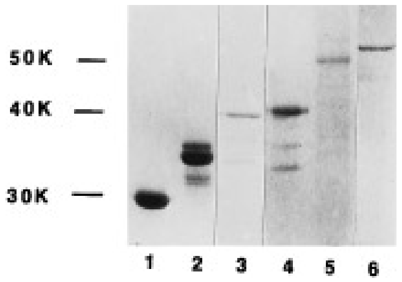

FIG. 1. Coommasie blue-stained SDS–PAGE of purified recombinant PUU N proteins. Lanes contain: (1) GST-c, (2) rN 1a, (3) rN 1b, (4) rN 3, (5) bac-PUU-N, (6) rN 2/3. Numbers at the left mark the mobilities of protein molecular mass standards in kilodaltons.

ylbenzidine substrate (Sigma) were used according to the manufacturer's instructions to detect specific antibody binding.

Animal immunization and PUU virus challenge

To assess immunogenicity as well as protection, 4- to 10-week-old bank voles, derived from a PUU virus-free colony established several years earlier with animals captured in Sweden, were immunized with purified truncated PUU virus N-GST fusion proteins, baculovirus PUU N (bac-PUU-N), or GST-c as a negative control. Bank voles were injected sc with 50 μg of specific proteins emulsified in Freund's complete adjuvant. The second and third injections were given with 3-week intervals, using the same amount of proteins in incomplete Freund's adjuvant and PBS, respectively. Serum antibody responses against PUU virus were measured 1 day before challenge by immunofluorescence assay (IFA; Lundkvist *et al.,* 1991) and focus reduction neutralization test (FRNT; Niklasson *et al.,* 1991). Bank voles were challenged sc 2 weeks after the last injection (8 weeks post-primary immunization) with 10^4 ID_{50} of PUU virus (strain Kazan). Animals were sacrificed at 21 days post challenge, and lungs were examined for presence of PUU virus-specific antigen by Hantavirus antigen-ELISA as previously described (Lundkvist *et al.,* 1995b).

RESULTS

Expression of truncated recombinant PUU virus N

The GST-fusion proteins rN 1a (aa 1–79), rN 1b (aa 1–118), rN 2b (aa 135–214), rN 2c (aa 135–327), and rN 3 (229–327) were cloned and expressed to high levels in *E. coli.* These GST-proteins and an earlier described construct rN 2/3 (aa 1–267), used for immunization of bank voles and in ELISA, were purified with glutathione sepharose beads in the presence of *N*-lauroylsarcosine with moderate to high yields (Fig. 1).

Epitope mapping with truncated rN fragments

The localization of B-cell epitopes in the N protein of PUU virus was investigated by a panel of bank vole Mabs, which recognize seven distinct antigenic sites on N, and with polyclonal sera from wild-trapped or experimentally infected bank voles. When assayed in immunoblotting with four truncated recombinant PUU virus N-fragments, the six Mabs 5E1, 5B5, 3G5, 1C12, 2E12, and 4E5 recognized all fragments covering the amino-terminal region of the protein, thus identifying six epitopes within the amino-terminal region (aa 1–79, Fig. 2A, Table 1). When two types of TUL virus rN proteins were assayed with Mabs that previously had been found cross-reactive with TUL virus (Plyusnin *et al.,* 1994), Mab 2E12 recognized rN Tot (aa 1–430) but not rN Eco (aa 1–61), indicating that this Mab reacted with a region located between aa 62 and 79 (Fig. 2B). A similar pattern was found for Mab 3G5, although the reactivity with rN Tot was weak. In addition to bac-PUU-N, Mab 3H9 reacted exclusively with two fragments, rN 2/3 (aa 1–267) and rN 3 (aa 229–327), which correlated well with the previously reported mapping of its epitope (N-a) to aa 251–260 (Lundkvist *et al.,* 1995a, Fig. 2C).

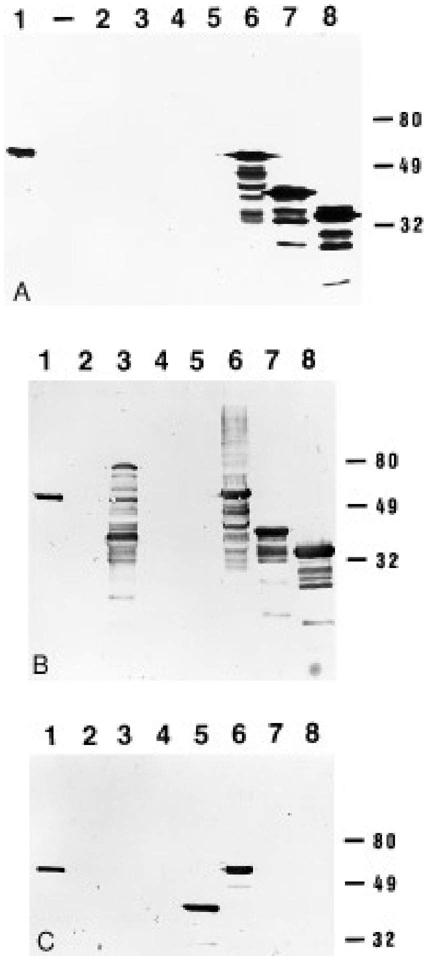

FIG. 2. Localization of Mab epitopes using truncated PUU virus N proteins in immunoblot assays. (A) Mab 5E1, (B) Mab 2E12, (C) Mab 3H9. Lanes contain: (1) bac-PUU-N, (2) Sf9 extract, (3) TUL rN Tot, (4) TUL rN Eco, (5) PUU rN 3, (6) PUU rN 2/3, (7) PUU rN 1b, (8) PUU rN 1a. Numbers at the right mark the mobilities of protein molecular mass standards in kilodaltons.

TABLE 1

Summary of Mab Reactivity in Immunoblotting with Truncated rN Proteins

Antigen	Mab (epitope) 3H9 (N-a)	5E1 (N-b)	5B5 (N-c)	3G5 (N-d)	1C12 (N-f)	2E12 (N-g)	4E5 (N-h)
PUU virus							
rN 1a (1–79)	–[a]	+	+	+	+	+	+
rN 1b (1–118)	–	+	+	+	+	+	+
rN 2/3 (1–267)	+	+	+	+	+	+	+
rN 3 (229–327)	+	–	–	–	–	–	–
bac-PUU-N (1–433)	+	+	+	+	+	+	+
Tula virus							
rN Eco (1–61)	–	–	–	–	+	–	+
rN Tot (1–430)	–	–	–	(+)	+	+	+

[a] +, positive reaction; (+), weak reaction; –, negative.

Pooled polyclonal sera from wild-trapped, PUU virus IgG-positive bank voles, from experimentally PUU virus-infected bank voles, and from bank voles immunized with bac-PUU-N were analyzed for IgG reactivity with truncated PUU rN fragments by ELISA (Table 2). The two sera obtained from infected animals were highly reactive with the amino-terminal fragments, in contrast to low or no reactivity with the fragments covering aa 135–327. Serum from animals immunized with bac-PUU-N reacted equally with all the different fragments except for rN 2b (aa 135–214). To examine the reactivity with the carboxy-terminal of N, sera were absorbed with rN 2/3 and 3 (equivalent to aa 1–327) before testing for reactivity to bac-PUU-N. Although the pooled sera from bac-PUU-N-immunized animals showed the highest reactivity, significant reactions were also seen with the serum pools from naturally and experimentally infected bank voles, suggesting the presence of B-cell epitopes between aa 328 and 433 (Table 2). No reactivity with the fragments rN 2/3 and 3 could be detected after the absorption.

TABLE 2

IgG Reactivity of Bank Vole Sera to Truncated Puumala Virus rN Proteins

Antigen	Serum PUU (wild)[a]	PUU (Kazan)[b]	Bac-PUU-N[c]	Nonimmune
rN 1a (1–79)	51,200[d]	51,200	12,800	<200
rN 1b (1–118)	12,800	12,800	3,200	<200
rN 2/3 (1–267)	51,200	3,200	12,800	<200
rN 2b (135–214)	<200	<200	200	<200
rN 2c (135–327)	200	200	12,800	<200
rN 3 (229–327)	<200	200	3,200	<200
C-terminal[e]	3,200	800	12,800	<200
bac-PUU-N (1–433)	3,200	3,200	12,800	<200

[a] Pooled sera ($n = 5$) from PUU virus IgG positive bank voles trapped in northern Sweden.

[b] Pooled sera ($n = 5$) drawn 3 weeks after experimental infection with PUU virus strain Kazan.

[c] Pooled sera ($n = 2$) from bank voles immunized with bac-PUU-N.

[d] Reciprocal end-point ELISA titers.

[e] Sera were preabsorbed with rN 2/3 and rN 3 (together equivalent to aa 1–327) before titration to bac-PUU-N.

PEPSCAN

In total, 141 overlapping 14-mer peptides of PUU virus (strain Sotkamo), covering the whole N in three-aa shifts, were used for epitope mapping using the PEPSCAN method. Several linear B-cell epitopes were identified over the entire N when a pool of polyclonal antisera, drawn from experimentally infected bank voles, were analyzed (Fig. 3A). Pooled sera from IgG-positive, wild-trapped bank voles revealed a similar pattern, although the reactivity was mainly directed to the first 50% (aa 1–210) of the protein (Fig. 3B). No unspecific reactivities were seen when a pool of sera from 5 noninfected colonized bank voles were analyzed (Fig. 3C).

Immunogenicity of rN and synthetic peptides

Sera from bank voles immunized with different rN fragments or bac-PUU-N were analyzed for the presence of antibodies to native PUU virus antigen. Pooled sera from bank voles infected with PUU virus, from noninfected animals, and from animals immunized with GST-c, were used as positive and negative controls, respectively. All rN fragments, as well as bac-PUU-N, evoked high titers of IgG reactive with native PUU N as determined by IFA. The result indicated the presence of several distinct immunogenic regions in the protein. The end-point titers are displayed in Table 3.

All except one of the pooled immune sera were found negative for neutralizing activity when assayed by FRNT

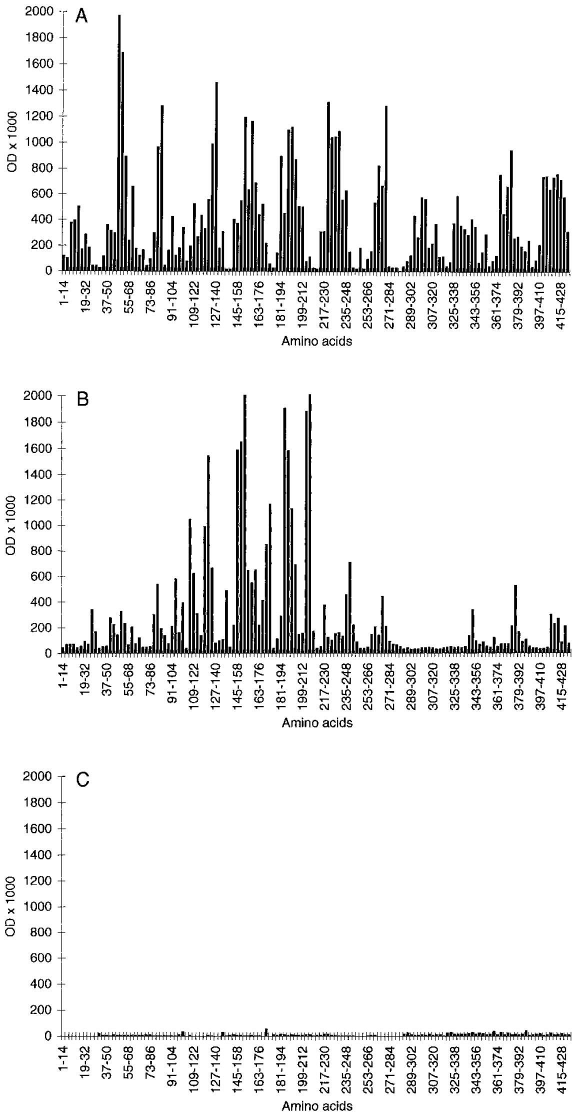

FIG. 3. PEPSCAN analysis of IgG reactivity to 14-mer peptides spanning PUU virus N with 3 aa overlap. Each bar displays the antibody reactivity (OD × 1000 at 405 nm). (A) Pooled sera ($n = 5$) from experimentally infected bank voles, (B) Pooled sera ($n = 3$) from PUU virus-immune bank voles trapped in northern Sweden, (C) Pooled sera ($n = 5$) from noninfected bank voles.

(Table 3). Sera from animals immunized with rN 2/3 neutralized PUU virus at the lowest dilution (1:40). Sera from bank voles experimentally or naturally infected with PUU virus showed neutralizing end-point titers of 1280.

A 30-aa synthetic peptide (P4, aa 241–270), covering the Mab epitope N-a (Lundkvist *et al.,* 1995a), located within a region of N previously defined as a major epitope in the human antibody response to PUU virus infection (Vapalahti *et al.,* 1995), was used for immunization of bank voles and Balb/C mice. When analyzed by IFA, pooled sera from bank voles and mice immunized with P4-KLH conjugate showed high reactivity with native PUU virus N with end-point titers of 800 and 6400, respectively. Bank voles immunized with P4 alone (i.e., without KLH-conjugation), raised N-specific IgG as well (titer 400).

Protection of bank voles from infection with PUU virus

An animal model that mimics the symptoms of PUU virus infection in humans has not been described. PUU virus causes a prolonged or persistent infection in bank voles, with no apparent pathogenicity. However, viral antigen can be detected routinely in selected organs of naturally or experimentally infected animals. To evaluate potential protective immune responses to PUU virus N, we developed a virus challenge model in bank voles, the major natural reservoir of this virus. We found that our stock of PUU virus strain Kazan, passaged exclusively in bank voles, infected all animals, as judged by N antigen detected in lungs, up to a dilution of $1{:}10^6$. In contrast, PUU virus strain Sotkamo, isolated and passaged in Vero E6, did not infect all animals reproducibly and viral antigen was found only sporadically. Therefore, for the protection experiments, animals were challenged with PUU virus strain Kazan diluted $1{:}10^3$, equivalent to 10^4 ID_{50}.

To evaluate the ability of the recombinant-expressed PUU virus N proteins to elicit a protective response in bank voles, the animals were given three immunizations with each rN protein. Serum antibody titers were measured after the third immunization (Table 3). Three weeks after the challenge with PUU virus, the animals were sacrificed and the lungs examined for the presence of PUU virus N antigen. None of the bank voles immunized with the bac-PUU-N preparation or the rN fragments 1a (aa 1–79), 1b (aa 1–118), and 2/3 (1–267) displayed N antigen in their lungs after challenge (Table 4). One of three animals immunized with rN 3 (aa 229–327) dis-

TABLE 3

Immune Responses to Puumala Virus in Bank Voles after Immunizations with Authentic, Expressed, or Synthetic Puumala Virus N

Immunogen	No. of animals	Reciprocal end-point titers		
		IFA	FRNT	G1/G2 ELISA
rN 1a (1–79)	2	6,400	<40	<200
	3	3,200	<40	<200
rN 1b (1–118)	3	3,200	<40	<200
rN 2/3 (1–267)	3	1,600	40	<200
rN 3 (229–327)	3	3,200	<40	<200
bac-PUU-N (1–433)	2	1,600	<40	<200
GST-c	5	<100	<40	<200
PUU virus (wild)[a]	5	1,600	1,280	6,400
PUU virus (Kazan)[b]	5	1,600	1,280	12,800
P4	3	400	<40	<200
P4-KLH	3	800	<40	<200
P4-KLH (mice)	3	6,400	<40	<200
Nonimmune control	2	<100	<40	<200

[a] Sera from PUU virus IgG positive wild bank voles trapped in northern Sweden.

[b] Sera drawn 3 weeks after experimental infection with PUU virus strain Kazan.

TABLE 4

Challenge of Bank Voles Immunized with Recombinant Puumala Virus N

Immunogen	Antigen in lungs	G1/G2 antibody
rN 1a (1–79)	0/5[a]	1/5[b] (12,800)[c]
rN 1b (1–118)	0/3	0/3
rN 2/3 (1–267)	0/3	0/3
rN 3 (229–327)	1/3	1/3 (⩾25,600)
bac-PUU-N (1–433)	0/8	0/8
GST-c	5/5	5/5 (12,800–⩾25,600)
Nonimmune control	8/8	8/8 (6,400–⩾25,600)

[a] Number of N-antigen positive/number of inoculated.

[b] Number of G1/G2-specific antibody positive/number of inoculated.

[c] Range of titer for all infected animals in each group.

played N antigen. All five control animals immunized with GST-c and all eight nonimmunized animals became N antigen positive after challenge.

In order to confirm our initial observation that the absence of N antigen in lungs of bank voles after viral challenge indicated protection, post-challenge sera were analyzed. Since IFA detects antibodies reactive with all the different viral structural proteins, and the prechallenge immunizations elicited high levels of N-reactive antibodies, we developed an ELISA based on human Mabs for specific detection of bank vole IgG reactive with the PUU virus envelope glycoproteins (G1 and G2). Protected animals should posses antibodies only to the N protein with which they were immunized, whereas animals that did become infected would exhibit demonstrable antibodies also to the viral glycoproteins. The assay was proven to detect only antibodies directed to the two PUU virus envelope glycoproteins when the antigen-specificity was evaluated by PUU virus N-, G1-, and G2-specific Mabs. No cross-reaction, due to unspecific binding to the solid-phase of N antigen, was shown with the N-specific Mab, which demonstrated disassociation of the detergent-disrupted and indirectly coated viral components. The detection limit of the assay was estimated to be less than 15 ng/ml of bank vole IgG by end-point titration of pooled G1- and G2-specific Mabs.

Individual post-challenge sera were examined for glycoprotein-specific antibodies by the G1/G2 ELISA. All animals immunized with the amino-terminal fragment rN 1b (aa 1–118), rN 2/3 (aa 1–267), or total bac-PUU-N, and lacking detectable N antigen, were found completely negative for glycoprotein-specific antibodies after challenge (Table 4). In contrast, nonimmunized animals or animals immunized with GST-c all showed high titers of glycoprotein-specific antibodies after challenge, as did PUU virus-immune wild-trapped bank voles (Tables 3 and 4). This confirmed that all animals immunized with proteins corresponding to aa 1–118 or larger amino-terminal fragments of PUU N were not infected with PUU virus after challenge. One of five animals immunized with the short amino-terminal fragment rN 1a (aa 1–79) developed glycoprotein-specific antibodies after challenge. The absence of N antigen in its lungs, however, suggested partial protection. One of the animals immunized with rN 3 (aa 229–327) displayed N antigen in its lungs and developed high levels of glycoprotein-specific antibodies after challenge. The results showed that immunization with fragments rN 1b or rN 3 may protect some animals from infection while not as effectively as immunizations with larger parts of PUU virus N.

DISCUSSION

The PUU virus nucleocapsid protein was shown to contain several B-cell epitopes recognized by the bank vole, the natural reservoir of this hantavirus. Six of seven previously defined epitopes, recognized by Mabs generated from a virus-infected bank vole, were mapped within the first 20% of the protein (aa 1–79), thereby indicating the amino-terminal region of PUU N as a major antigenic region. The localization of four Mab-epitopes within aa 1–61, and two between aa 62–79, correlated well with our earlier mapping data, based on additivity and competitive ELISAs, together with reactivity patterns with various hantavirus strains, which suggested that several of the epitopes were partially or completely overlapping (Lundkvist *et al.,* 1991; Lundkvist and Niklasson, 1992).

Polyclonal sera from naturally or experimentally infected bank voles revealed the presence of B-cell epitopes over the entire N. Although sera from infected animals were nonreactive with the rN 2 b fragment (aa 135–214), PEPSCAN data indicated the presence of antigenic domains also within this region. The suggestion that the amino-terminal region constitutes the major antigenic target in PUU virus-infected bank voles, as indicated by the end-point titers to the different N-fragments, is in concordance with the Mab-epitope mapping. In contrast, immunizations with complete N (bac-PUU-N) gave rise to similar IgG levels to all different fragments, except rN 2b. Although the PEPSCAN data and the IgG end-point titers to the different rN fragments correlated to some extent, discrepancies were found. The latter method detected reactivities primarily to the amino-terminus, while PEPSCAN detected epitopes throughout the protein. This could be due to several reasons; e.g., the low reactivity seen with the middle part of N using rN fragments can be explained either by epitopes hidden in the protein and/or that these linear epitopes, detected by PEPSCAN, are not properly presented in *E. coli*-expressed rN. We have previously shown that the epitopes N-a and N-e are not recognized in rN expressed as β-gal-N fusion protein in contrast to the baculovirus recombinant where all examined N-epitopes were detected (Vapalahti *et al.,* 1996). N-a, but not N-e, was properly expressed in the GST fusion-proteins used in this study (data not shown).

These results suggested that, although highly immunogenic and antigenic domains are present throughout N, the IgG responses in infected bank voles are mainly directed to the amino-terminus. Studies on the human IgG response to PUU virus N have shown a similar pattern, i.e., data based on truncated N proteins indicated the amino-terminal part as the major antigenic region although PEPSCAN data revealed the presence of antigenic domains also in other parts of the protein (Gött *et al.,* 1991; Lundkvist *et al.,* 1995a; Vapalahti *et al.,* 1992; 1995; F. Elgh and Å. Lundkvist, unpublished). Similarly, it has been shown for Sin Nombre virus that the major domain for the humoral reactivity resides within the amino-terminus of an *E. coli*-expressed N protein (Jenison *et al.,* 1994; Yamada *et al.,* 1995).

Examination by IFA revealed that all the different rN fragments elicited significant levels of IgG levels reactive with native PUU N. The highly immunogenic nature of the amino-terminal part was further confirmed by the relatively high antibody titers to native N evoked in animals immunized with rN 1a (aa 1–79); none of the pooled sera raised to the larger rN fragments or to the entire N (i.e., bac-PUU-N or during viral infection) showed higher titers to native N. The peptide P4, conjugated to KLH, elicited high antibody responses to native N both in bank voles and mice. Interestingly, bank voles immunized with P4 alone (i.e., without KLH-conjugation), raised N-specific IgG as well, indicating the presence of at least one specific T-cell epitope within aa 241–270.

The absence of known HFRS-like disease in animals made it impossible to evaluate the ability of our recombinant proteins to moderate disease; however, a more stringent assay for measurement of protection from PUU virus infection was developed. As demonstrated by virus challenge experiments, none of the animals immunized with the amino-terminal fragments or with complete recombinant N (bac-PUU-N) displayed N antigen in their lungs even after challenge with 10^4 ID_{50} of PUU virus. When postchallenge sera were analyzed, only one of the animals (immunized with the shortest amino-terminal fragment), did not appear to be completely protected as judged by the presence of glycoprotein-specific antibodies. This confirmed that all animals immunized with proteins corresponding to aa 1–118, or with larger amino-terminal fragments of PUU N, were completely protected against infection. In line with this, HTN virus recombinant N has previously been shown to protect hamsters or suckling mice from HTN virus infection (Schmaljohn *et al.,* 1990; Yoshimatsu *et al.,* 1993). The results from our animal model also emphasize the importance of investigating not only the presence of viral antigen, but also the antibody responses.

The envelope glycoproteins are presumed to be the major elements involved in induction of immunity to hantaviruses. This assumption is based on passive protection experiments and by the neutralizing activity detected *in vitro* for Mabs directed to G1 and G2, but not to N (Dantas *et al.,* 1986; Arikawa *et al.,* 1989; Zhang *et al.,* 1989; Schmaljohn *et al.,* 1990; Arikawa *et al.,* 1992; Lundkvist and Niklasson, 1992). The significance of the N-specific antibody response *in vivo* is, however, not yet completely understood. A Mab specific for HTN virus N has been shown to protect from virus infection and N-specific polyclonal sera to significantly increase the time before death in a mouse model (Yoshimatsu *et al.,* 1993). N-specific Mabs have been shown to partially protect bank voles from PUU virus infection (Lundkvist *et al.,* unpublished). Therefore, it is likely that the explanation to the reported absence of neutralizing activity for all hantavirus N-specific Mabs, as determined by NT or FRNT, is due to the use of *in vitro* systems, which do not necessarily reflect the situation *in vivo.* Accordingly, the humoral response to N may, in addition to the glycoprotein-specific antibody response, be of importance for the immunity, e.g., via antibody-dependent cell-mediated cytotoxity and complement-mediated cytolysis.

Previous reports have suggested that a cell-mediated response to HTN virus is involved in protection (Asada *et al.,* 1988, 1989; Yoshimatsu *et al.,* 1993). Since all except one of the pooled prechallenge sera in our experiments were found negative for neutralizing antibodies when assayed by FRNT, the protection, evoked by the different regions of PUU virus N, was interpreted as mainly dependent on cell-mediated immune responses. Additional information is required to further define the important aspects of the immunity to PUU virus. Our data suggest, regardless of the mechanism(s), that our recombinant-expressed N proteins are clearly capable of inducing a response that can protect animals from infection after challenge with high doses of PUU virus.

In conclusion, our results revealed the location of several B-cell epitopes in PUU virus N and indicated that the amino-terminus is the major antigenic domain in infected bank voles. Our data further demonstrated the feasibility of using expressed or synthetic fragments of PUU virus N to elicit high antibody responses to the native protein and indicated the importance of N, especially the amino-terminus, in the course of protective immunity. This study may also provide a basis for future experiments concerning recombinant-expressed PUU virus proteins as potential human vaccines.

ACKNOWLEDGMENTS

We thank Mari Jonsson for excellent technical assistance. This project was in part supported by grants from the Swedish Medical Research Council (Project No 11230), and Sigrid Jusélius Foundation, Helsinki, Finland.

REFERENCES

Antic, D., Lim, B.-U., and Kang, C. Y. (1991). Nucleotide sequence and coding capacity of the large (L) genomic RNA segment of Seoul 80-39 virus, a member of the hantavirus genus. *Virus Res.* 19, 59–66.

Arikawa, J., Schmaljohn, A. L., Dalrymple, J. M., and Schmaljohn, C. S. (1989). Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. *J. Gen. Virol.* 70, 615–624.

Arikawa, J., Yao, J.-S., Yoshimatsu, K., Takashima, I., and Hashimoto, N. (1992). Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. *Arch. Virol.* 126, 271–281.

Asada, H., Tamura, M., Kondo, K., Dohi, Y., and Yamanishi, K. (1988). Cell-mediated immunity to virus causing haemorrhagic fever with renal syndrome: generation of cytotoxic T lymphocytes. *J. Gen. Virol.* 69, 2179–2188.

Asada, H., Balachandra, K., Tamura, M., Kondo, K., and Yamanishi, K. (1989). Cross-reactive immunity among different serotype of virus causing haemorrhagic fever with renal syndrome. *J. Gen. Virol.* 70, 819–825.

Berzins, K., Perlman, H., Wåhlin, B., Carlsson, J., Wahlgren, M., Udomsangpetch, R., Björkman, A., Patarroyo, M. E., and Perlman, P. (1986). Rabbit and human antibodies to a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf 155, react with the native protein and inhibit merozoite invasion. *Proc. Natl. Acad. Sci. USA* 83, 1065–1069.

Brummer-Korvenkontio, M., Vaheri, A., Hovi, T., von Bonsdorff, C.-H., Vuorimies, J., Manni, T., Penttinen, K., Oker-Blom, N., and Lähdevirta, J. (1980). Nephropathia epidemica: Detection of antigen in bank voles and serologic diagnosis of human infection. *J. Infect. Dis.* 141, 131–134.

Chu, Y. K., Rossi, C., LeDuc, J. W., Lee, H. W., Schmaljohn, C. S., and Dalrymple, J. M. (1994). Serological relationships among viruses in the *Hantavirus* genus, family *Bunyaviridae. Virology* 198, 196–204.

Dantas, J. R., Jr., Okuno, Y., Asada, H., Tamura, M., Takahashi, M., Tanishita, O., Takahashi, Y., Kurata, T., and Yamanishi, K. (1986). Characterization of glycoproteins of viruses causing hemorrhagic fever with renal syndrome (HFRS) using monoclonal antibodies. *Virology* 151, 379–384.

Dzagurova, T., Tkachenko, E., Slonova, R., Ivanov, L., Ivanidze, E., Markeshin, S., Dekonenko, A., Niklasson, B., and Lundkvist, Å. (1995). Antigenic relationships of hantavirus strains analysed by monoclonal antibodies. *Arch. Virol.* 140, 1763–1773.

Elliott, R. M. (1990). Molecular biology of the Bunyaviridae. *J. Gen. Virol.* 71, 501–522.

Frangioni, J., and Neel, B. (1993). Solubilisation and purification of enzymatically active glutathione s-transferase (pGEX) fusion proteins. *Anal. Biochem.* 210, 179–187.

Gavrilovskaya, I. N., Chumakov, M. P., Apekina, N. S., Rylceva, E. V., Martiyanova, L. I., Gorbachkova, E. A., Bernshtein, A. D., Zakharova, M. A., and Boiko, V. A. (1983). Adaption to laboratory and wild animals of the haemorrhagic fever with renal syndrome virus present in the foci of European U.S.S.R. *Arch. Virol.* 77, 87–90.

Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbick, G., and Schoffs, P. G. (1987). Strategies for epitope analysis using peptide synthesis. *J. Immunol. Methods* 102, 259–274.

Gött, P., Zöller, L., Yang, S., Stohwasser, R., Bautz, E. K., and Darai, G. (1991) Antigenecity of hantavirus nucleocapsid proteins expressed in *E. coli. Virus Res.* 19, 1–15.

Harlow, E., and Lane, D. (1988). "Antibodies: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hjelle, B., Jenison, S., Torrez-Martinez, N., Yamada, T., Nolte, K., Zumwalt, R., MacInnes, K., and Myers, G. (1994a). A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: Evolutionary relationships to known hantaviruses. *J. Virol.* 68, 592–506.

Hjelle, B., Chaves-Giles, F., Torrez-Martinez, N., Yates, T., Sarisky, S., Webb, J., and Ascher, M. (1994b). Genetic identification of a novel hantavirus of the harvest mouse *Reithrodontomys megalotis. J. Virol.* 68, 6751–6754.

Jenison, S., Yamada, T., Morris, C., Andersson, B., Torrez-Martinez, N., Keller, N., and Hjelle, B. (1994). Characterization of human antibody responses to Four Corners Hantavirus infections among patients with Hantavirus pulmonary syndrome. *J. Virol.* 68, 3000–3006.

Lundkvist, Å., Fatouros, A., and Niklasson, B. (1991). Antigenic variation of European haemorrhagic fever with renal syndrome virus strains characterized using bank vole monoclonal antibodies. *J. Gen. Virol.* 72, 2097–2103.

Lundkvist, Å., and Niklasson, B. (1992). Bank vole monoclonal antibodies against Puumala virus envelope glycoproteins: Identification of epitopes involved in neutralization. *Arch. Virol.* 126, 93–105.

Lundkvist, Å., Hörling, J., Athlin, L., Rosén, A., and Niklasson, B. (1993a). Neutralizing human monoclonal antibodies against Puumala virus, causative agent of nephropathia epidemica: A novel method using antigen-coated magnetic beads for specific B-cell isolation. *J. Gen. Virol.* 74, 1303–1310.

Lundkvist, Å., Scholander, C., and Niklasson, B. (1993b). Anti-idiotypic antibodies against Puumala virus glycoprotein-specific monoclonal antibodies inhibit virus infection in cell culture. *Arch. Virol.* 132, 255–265.

Lundkvist, Å., and Niklasson, B. (1994). Hemorrhagic fever with renal syndrome and other hantavirus infections. *Rev. Med. Virol.* 4, 177–184.

Lundkvist, Å., Björsten, S., Niklasson, B., and Ahlborg, N. (1995a). Mapping of B-cell determinants in the nucleocapsid protein of Puumala virus: Definition of epitopes specific for the acute immunoglobin G recognition in humans. *Clin. Diag. Lab. Immunol.* 2, 82–86.

Lundkvist, Å., Hörling, J., Björsten, S., and Niklasson, B. (1995b). Sensitive detection of hantaviruses by biotin-strepavidin enhanced immunoassays based on bank vole monoclonal antibodies. *J. Virol. Methods* 52, 75–86.

Morzunov, S. P., Feldmann, H., Spiropoulou, C. F., Semenova, V. A., Rollin, P. E., Ksiazek, T. G., Peters, C. J., and Nichol, S. (1995). A newly recognized virus associated with afatal case of hantavirus pulmonary syndrome in Louisiana. *J. Virol.* 69, 1980–1983.

Niklasson, B., Jonsson, M., Lundkvist, Å., Hörling, J., and Tkachenko, E. (1991). European isolates of viruses causing hemorrhagic fever with renal syndrome compared by neutralization test. *Am. J. Trop. Med. Hyg.* 45, 660–665.

Nichol, S. T., Spiropoulou, C. F., Morzunov, S., Rollin, P. E., Ksiazek, T. G., Feldmann, H., Sanchez, A., Childs, J., Zaki, S., and Peters, C. J. (1993). Genetic identification of a hantavirus associated with a outbreak of acute respiratory illness. *Science* 262, 914–917.

Plyusnin, A., Vapalahti, O., Lankinen, H., Lehväslaiho, H., Apekina, N., Myasnikov, Y., Kallio-Kokko, H., Henttonen, H., Lundkvist, Å., Brummer-Korvenkontio, M., Gavrilovskaya, I., and Vaheri, A. (1994). Tula virus: A newly detected hantavirus carried by European common voles. *J. Virol.* 68, 7833–7838.

Rollin, P. E., Ksiazek, T. G., Elliott, L. H., Ravkov, E. V., Martin, M. L., Morzunov, S., Livingstone, W., Monroe, M., Glass, G., Ruo, S., Khan, A. S., Childs, J. E., Nichol, S. T., and Peters, C. J. (1995). Isolation of Black Creek Canal virus, a new hantavirus from *Sigmodon hispidus* in Florida. *J. Med. Virol.* 46, 35–39.

Schmaljohn, C. S., and Dalrymple, J. M. (1983). Analysis of Hantaan virus RNA: Evidence of a new genus of *Bunyaviridae. Virology* 131, 482–491.

Schmaljohn, C. S., Hasty, S. E., Dalrymple, J. M., LeDuc, J. W., Lee, H. W., von Bonsdorff, C.-H., Brummer-Korvenkontio, M., Vaheri, A., Tsai, T. F., Regnery, H. L., Goldgaber, D., and Lee, P. W. (1985). Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. *Science* 227, 1041–1044.

Schmaljohn, C. S., Chu, Y-K., Schmaljohn, A. L., and Dalrymple, J. M. (1990). Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J. Virol.* 64, 3162–3170.

Schmaljohn, A. L., Li., D., Negley, D. L., Bressler, D. S., Turell, M. J., Korch, G. W., Ascher, M. S., and Schmaljohn, C. S. (1995). Isolation and initial characterization of a newfound hantavirus from California. *Virology* 206, 963–972.

Spiropoulou, C., Morzunov, S., Feldmann, H., Sanchez, A., Peters, C. J., and Nichol, S. (1994). Genome structure and variability of a virus causing Hantavirus pulmonary syndrome. *Virology* 200, 715–723.

Studier, F. W., Rosenberg, A., Dunn, J. J., and Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60–89.

Vapalahti, O., Kallio-Kokko, H., Salonen, E.-M., Brummer-Korvenkontio, M., and Vaheri, A. (1992). Cloning and sequencing of Puumala virus Sotkamo strain S and M RNA segments: Evidence for strain variation in hantaviruses and expression of the nucleocapsid protein. *J. Gen. Virol.* 73, 829–838.

Vapalahti, O., Kallio-Kokko, H., Närvänen, A., Julkunen, I., Lundkvist, Å., Plyusnin, A., Lehväslaiho, H., Brummer-Korvenkontio, M., Vaheri, A., and Lankinen, H. (1995). Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. *J. Med. Virol.* 46, 293–303.

Vapalahti, O., Lundkvist, Å., Kallio-Kokko, H., Paukku, K., Julkunen, I., Lankinen, H., and Vaheri, A. (1996). Antigenic properties and diagnostic potential of Puumala virus nucleocapsid protein expressed in insect cells. *J. Clin. Microbiol.* 34, 119–125.

Xiao, S.-Y., LeDuc, J. W., Chu, Y. K., and Schmaljohn, C. S. (1994). Phylogenetic analysis of virus isolates in the genus *Hantaviruses,* family *Bunyaviridae. Virology* 198, 295–217.

Yamada, Y., Hjelle, B., Lanzi, R., Morris, C., Andersson, B., and Jenison, S. (1995). Antibody responses to Four Corners hantavirus infections in the Deer mouse (*Peromyscus maniculatus*): Identification of an immunodominant region of the viral nucleocapsid protein. *J. Virol.* 69, 1939–1943.

Yanagihara, R., and Gajdusek, D. C. (1988). Hemorrhagic fever with renal syndrome: A historical perspective and review of recent advances. *In* Handbook on Viral and Rickettsial Hemorrhagic Fevers" (J. H. S. Gear, Ed.), pp. 151–188. CRC Press, Boca Raton.

Yoshimatsu, K., Yoo, Y.-C., Yoshida, R., Ishihara, C., Azuma, I., and Arikawa, J. (1993). Protective immunity of Hantaan virus nucleocapsid and envelope protein studied using baculovirus-expressed proteins. *Arch. Virol.* 130, 365–376.

Zhang, X.-K., Takashima, I., and Hashimoto, N. (1989). Characteristics of passive immunity against hantavirus infection in rats. *Arch. Virol.* 105, 235–246.