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(54) Title: VACCINE AGAINST HANTAVIRUS

(57) Abstract

Virus vaccine against Hantaviruses, especially Puumala virus, which comprises, an immunizing component, at least one member of the group consisting of: a) a recombinant protein having the amino-acid sequence of the figure; b) fragments of said protein which comprise B-cell and/or T-cell epitopes; and c) amino-acid sequences which are at least 80 % homologous to the sequences of a) or b) and which comprise B-cell and/or T-cell epitopes, are disclosed.

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VACCINE AGAINST HANTAVIRUS

The present invention relates to vaccines against hantaviruses, especially Puumala virus.

BACKGROUND OF THE INVENTION

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 (Xiao et al., 1994). In addition, nucleotide sequence and immunological data on five potentially new serotype viruses, Tula (TUL), El Moro Canyon, Khabarovsk, Bayou, and Black Creek Canal have been reported. Small mammals, mainly rodents, are the natural reservoirs of hantaviruses and transmission to humans occurs via aerosolized animal excreta.

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%-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).

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) (Antic et al., 1991). The envelope glycoproteins 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 (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., 1995a). With a panel of PUU virus bank vole Mabs, reactive with 8 distinct epitopes on N, only one epitope could be defined when overlapping 10-mer amino acid peptides were used (Lundkvist et al., 1995a).

DESCRIPTION OF THE INVENTION

We have now surprisingly found that immunity against Puumala virus infection can be elicited by the administration of a recombinant protein having the amino-acid sequence of the Puumala virus nucleocapsid protein (N) disclosed in Fig.1 (Vapalahti et al. 1992), and some truncated fragments thereof (disclosed in Figs. 2-6).

Thus, the present invention is directed to virus vaccines, which comprise, as an immunizing component, at least one member of the group consisting of

a) a recombinant protein having the amino-acid sequence of Fig.1,

b) fragments of said protein which comprise B-cell and/or T-cell epitopes and,

c) amino-acid sequences which are at least 80% homologous to the sequences of a) or b) and which comprise B-cell and/or T-cell epitopes.

It is believed that some amino-acid substitutions, deletions, and extensions may occur in the above defined protein a) and fragments b), while such homologous amino-acid sequences defined in c) will still provoke the same type of protective immunity. The closer the homologous sequence is to the parent sequence the more likely the properties are the same. Thus, homologies of 95%, 90% and 85% are preferred.

In the virus vaccines of the invention the antigenic proteins or peptides are optionally coupled to adjuvants. The adjuvants should be selected from pharmaceutically acceptable adjuvants accepted for use in human vaccines by the health authorities, e.g. FDA.

Fragments of the protein a) which can impart at least partial immunity have been disclosed in Fig.2, Fig.3, Fig.4, Fig.5, and Fig.6.

To further define the localization of B-cell epitopes recognized by the natural reservoir of PUU virus, the bank vole, we chose to use such 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 *E.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 mM 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 x 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 (s.c.), 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 ordered from and 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, 1995b). Briefly, the antigen consisted of an extract of *Spodoptera frugiperda* (Sf9) cells containing approximately 35% of bac-PUU-N, solubilised 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 BamHI and Eco RI sites of pGEX2T vector (Pharmacia, Uppsala, Sweden) as follows: Construct rN 1a coding for aa 1-79 with primers having the following nucleotide sequenses:

A: (TTTGAATTCTCT**AGATCT**GGAATGAGTGACTTGACAGA) and
B: (TTT**GAATTC**TTGGATCCAGTCGGTTAAGTAGGTTTAGTA),
Construct rN 1b coding for aa 1-118 with primers A and
C: (TTT**GAATTC**GTCGACTCAATCTGCTGTTTGGCCACTTG),
Construct rN 3 coding for aa 229-327 and six extra aa from the polylinker area with primers
D: (TTTGTCGAC**GGATCC**AAGGATTGGTCTGAGAGAA) and
E: (TTT**GAATTC**GTCGACTCAGCAACATAGATACATGTTGG),
Construct rN 2b coding for aa 135-214 with primers
F: (TTTGTCGAC**GGATCC**AAAGCTTTATACATGTCTC and
G: (TTT**GAATTC**GTCGACTCAGTTAGCAACCTGGATCTGAG),
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, 1995a) 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, Sweden). Briefly, overnight cultures of transformed BL21 bacteria (Studier et al, 1990) were diluted 1/100 and grown for 2 h at 37°C, induced with 0.5 mM IPTG for 3h 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 et al. (1993). The protein was eluted from the beads by 25mM reduced glutathione (Boehringer Mannheim, Mannheim, Germany) and dialysed.

Immunoblotting

Bacterial cell suspensions were suspended in Laemmli's sample buffer, proteins separated in SDS-PAGE and transferred to Immobilon filters (Millipore, Bedford, MA). After blocking, filters were subsequently incubated with Mabs (1µg/ml) overnight at 4°C, followed by rabbit anti-mouse peroxidase-conjugate (Dakopatts,

Denmark), diluted 1:400, for 1h at 20°C. 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 3 amino acids; Vapalahti et al., 1995a) 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 non-purified proteins, or non-purified bac-PUU-N (diluted 1:1000), were adsorbed to microtiter plates overnight at 4°C. Unsaturated protein-binding sites were blocked by addition of 3% bovine serum albumine (BSA) in PBS for 1h. Serial dilutions of sera in ELISA buffer (PBS with 0.5% BSA and 0.05% Tween-20) were pre-incubated with 1% GST-c for 1 h before addition to plates. Adsorbed sera were incubated for 1 h 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°C 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 pre-absorbed 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 h at 20°C.

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°C. All the following incubations were performed for 1 h at 37°C 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 two-fold dilutions of sera (starting at 1:200), were added to the plates. ALP-labelled 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 h. Peroxidase-conjugated streptavidin (Sigma), followed by tetramethylbenzidine 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 s.c. 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 s.c. 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).

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 (aa 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.

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, 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 62-79. 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).

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 328-433 (Table 2). No reactivity with the fragments rN 2/3 and 3 could be detected after the absorption.

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. 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. No unspecific reactivities were seen when a pool of sera from 5 non-infected colonized bank voles were analyzed.

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 non-infected 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 (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., 1995a), 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 out of three animals immunized with rN 3 (aa 229-327) displayed N antigen. All five control animals immunized with GST-c and all eight non-immunized 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 pre-challenge 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, non-immunized 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 out 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.

Conclusions

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 non-reactive 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., 1995b). 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 revaled the presence of antigenic domains also in other parts of the protein (Lundkvist et al., 1995a; Vapalahti et al., 1992; 1995a; 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 post-challenge 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 (Schmaljohn et al., 1990; 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., 1987, 1988; Yoshimatsu et al., 1993). Since all except one of the pooled pre-challenge 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.

Table 1. Summary of Mab reactivity in immunoblotting with truncated rN proteins.

	Mab (epitope)						
Antigen	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

Table 2. IgG reactivity of bank vole sera to truncated Puumala virus rN proteins.

	Serum			
Antigen	PUU (wild)[a]	PUU (Kazan)[b]	Bac-PUU-N[c]	Non-immune
rN 1a (1-79)	51200[d]	51200	12800	<200
rN 1b (1-118)	12800	12800	3200	<200
rN 2/3 (1-267)	51200	3200	12800	<200
rN 2b (135-214)	<200	<200	200	<200
rN 2c (135-327)	200	200	12800	<200
rN 3 (229-327)	<200	200	3200	<200
C-terminal[e]	3200	800	12800	<200
bac-PUU-N (1-433)	3200	3200	12800	<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 pre-absorbed with rN 2/3 and rN 3 (together equivalent to aa 1 - 327) before titration to bac-PUU-N.

Table 3. Immune responses to Puumala virus in bank voles after immunizations with authentic, expressed or synthetic Puumala virus N.

		Reciprocal end-point titers		
Immunogen	No. of animals	IFA	FRNT	G1/G2 ELISA
rN 1a (1-79)	2	6400	< 40	< 200
	3	3200	< 40	< 200
rN 1b (1-118)	3	3200	< 40	< 200
rN 2/3 (1-267)	3	1600	40	< 200
rN 3 (229-327)	3	3200	< 40	< 200
bac-PUU-N (1-433)	2	1600	< 40	< 200
GST-c	5	<100	< 40	< 200
PUU virus (wild)[a]	5	1600	1280	6400
PUU virus (Kazan)[b]	5	1600	1280	12800
P4	3	400	< 40	< 200
P4-KLH	3	800	< 40	< 200
P4-KLH (mice)	3	6400	< 40	< 200
Non-immune 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] (12800)[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 (≥25600)
bac-PUU-N (1-433)	0/8	0/8
GST-c	5/5	5/5 (12800 - ≥25600)
Non-immune control	8/8	8/8 (6400 - ≥25600)

[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.

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CLAIMS

1. Virus vaccine, which comprises, as an immunizing component, at least one member of the group consisting of

a) a recombinant protein having the amino-acid sequence of Fig.1,

b) fragments of said protein which comprise B-cell and/or T-cell epitopes, and

c) amino-acid sequences which are at least 80% homologous to the sequences of a) or b) and which comprise B-cell and/or T-cell epitopes.

2. Virus vaccine according to claim 1, wherein said member is coupled to an adjuvant.

3. Virus vaccine according to claim 1 or 2, wherein said member is selected from the members b) which have the amino-acid sequences of Fig.2, Fig.3, Fig.4, Fig.5, and Fig.6.

FIG. 1

bac-PUU-Asn (1-433)

```
Met Ser Asp Leu Thr Asp Ile Gln Glu Asp Ile Thr Arg His Glu Gln
                5                  10                  15
Gln Leu Ile Val Ala Arg Gln Lys Leu Lys Asp Ala Glu Arg Ala Val
            20                  25                  30
Glu Val Asp Pro Asp Asp Val Asn Lys Asn Thr Leu Gln Ala Arg Gln
        35                  40                  45
Gln Thr Val Ser Ala Leu Glu Asp Lys Leu Ala Asp Tyr Lys Arg Arg
    50                  55                  60
Met Ala Asp Ala Val Ser Arg Lys Lys Met Asp Thr Lys Pro Thr Asp
65                  70                  75                  80
Pro Thr Gly Ile Glu Pro Asp Asp His Leu Lys Glu Arg Ser Ser Leu
                85                  90                  95
Arg Tyr Gly Asn Val Leu Asp Val Asn Ala Ile Asp Ile Glu Glu Pro
            100                 105                 110
Ser Gly Gln Thr Ala Asp Trp Tyr Thr Ile Gly Val Tyr Val Ile Gly
        115                 120                 125
Phe Thr Leu Pro Ile Ile Leu Lys Ala Leu Tyr Met Leu Ser Thr Arg
    130                 135                 140
Gly Arg Gln Thr Val Lys Glu Asn Lys Gly Thr Arg Ile Arg Phe Lys
145                 150                 155                 160
Asp Asp Thr Ser Phe Glu Asp Ile Asn Gly Ile Arg Arg Pro Lys His
                165                 170                 175
Leu Tyr Val Ser Met Pro Thr Ala Gln Ser Thr Met Lys Ala Glu Glu
            180                 185                 190
Leu Thr Pro Gly Arg Phe Arg Thr Ile Val Cys Gly Leu Phe Pro Thr
        195                 200                 205
Gln Ile Gln Val Arg Asn Ile Met Ser Pro Val Met Gly Val Ile Gly
    210                 215                 220
Phe Ser Phe Phe Val Lys Asp Trp Ser Glu Arg Ile Arg Glu Phe Met
225                 230                 235                 240
Glu Lys Glu Cys Pro Phe Ile Lys Pro Glu Val Lys Pro Gly Thr Pro
                245                 250                 255
Ala Gln Glu Ile Glu Met Leu Lys Arg Asn Lys Ile Tyr Phe Met Gln
            260                 265                 270
Arg Gln Asp Val Leu Asp Lys Asn His Val Ala Asp Ile Asp Lys Leu
        275                 280                 285
Ile Asp Tyr Ala Ala Ser Gly Asp Pro Thr Ser Pro Asp Asn Ile Asp
    290                 295                 300
Ser Pro Asn Ala Pro Trp Val Phe Ala Cys Ala Pro Asp Arg Cys Pro
305                 310                 315                 320
Pro Thr Cys Ile Tyr Val Ala Gly Met Ala Glu Leu Gly Ala Phe Phe
                325                 330                 335
Ser Ile Leu Gln Asp Met Arg Asn Thr Ile Met Ala Ser Lys Thr Val
            340                 345                 350
Gly Thr Ala Glu Glu Lys Leu Lys Lys Lys Ser Ser Phe Tyr Gln Ser
        355                 360                 365
Tyr Leu Arg Arg Thr Gln Ser Met Gly Ile Gln Leu Asp Gln Arg Ile
    370                 375                 380
Ile Leu Leu Phe Met Leu Glu Trp Gly Lys Glu Met Val Asp His Phe
385                 390                 395                 400
His Leu Gly Asp Asp Met Asp Pro Glu Leu Arg Gly Leu Ala Gln Ala
                405                 410                 415
Leu Ile Asp Gln Lys Val Lys Glu Ile Ser Asn Gln Glu Pro Leu Lys
            420                 425                 430
Thr
```

FIG. 2

rN 3 (229-327)

```
Lys Asp Trp Ser Glu Arg Ile Arg Glu Phe Met Glu Lys Glu Cys Pro
                5                  10                  15
Phe Ile Lys Pro Glu Val Lys Pro Gly Thr Pro Ala Gln Glu Ile Glu
            20                  25                  30
Met Leu Lys Arg Asn Lys Ile Tyr Phe Met Gln Arg Gln Asp Val Leu
        35                  40                  45
Asp Lys Asn His Val Ala Asp Ile Asp Lys Leu Ile Asp Tyr Ala Ala
    50                  55                  60
Ser Gly Asp Pro Thr Ser Pro Asp Asn Ile Asp Ser Pro Asn Ala Pro
65                  70                  75                  80
Trp Val Phe Ala Cys Ala Pro Asp Arg Cys Pro Pro Thr Cys Ile Tyr
                85                  90                  95
Val Ala
```

FIG. 3

rN 2/3 (1-267)

```
Met Ser Asp Leu Thr Asp Ile Gln Glu Asp Ile Thr Arg His Glu Gln
                5                  10                  15
Gln Leu Ile Val Ala Arg Gln Lys Leu Lys Asp Ala Glu Arg Ala Val
            20                  25                  30
Glu Val Asp Pro Asp Asp Val Asn Lys Asn Thr Leu Gln Ala Arg Gln
        25                  30                  35
Gln Thr Val Ser Ala Leu Glu Asp Lys Leu Ala Asp Tyr Lys Arg Arg
    40                  45                  50
Met Ala Asp Ala Val Ser Arg Lys Lys Met Asp Thr Lys Pro Thr Asp
55                  60                  65                  70
Pro Thr Gly Ile Glu Pro Asp Asp His Leu Lys Glu Arg Ser Ser Leu
                75                  80                  85
Arg Tyr Gly Asn Val Leu Asp Val Asn Ala Ile Asp Ile Glu Glu Pro
            90                  95                  100
Ser Gly Gln Thr Ala Asp Trp Tyr Thr Ile Gly Val Tyr Val Ile Gly
        105                 110                 115
Phe Thr Leu Pro Ile Ile Leu Lys Ala Leu Tyr Met Leu Ser Thr Arg
    120                 125                 130
Gly Arg Gln Thr Val Lys Glu Asn Lys Gly Thr Arg Ile Arg Phe Lys
135                 140                 145                 150
Asp Asp Thr Ser Phe Glu Asp Ile Asn Gly Ile Arg Arg Pro Lys His
                155                 160                 165
Leu Tyr Val Ser Met Pro Thr Ala Gln Ser Thr Met Lys Ala Glu Glu
            170                 175                 180
Leu Thr Pro Gly Arg Phe Arg Thr Ile Val Cys Gly Leu Phe Pro Thr
        185                 190                 195
Gln Ile Gln Val Arg Asn Ile Met Ser Pro Val Met Gly Val Ile Gly
    200                 205                 210
Phe Ser Phe Phe Val Lys Asp Trp Ser Glu Arg Ile Arg Glu Phe Met
215                 220                 225                 230
Glu Lys Glu Cys Pro Phe Ile Lys Pro Glu Val Lys Pro Gly Thr Pro
                235                 240                 245
Ala Gln Glu Ile Glu Met Leu Lys Arg Asn Lys
            250                 255
```

FIG. 4

rN 1b(1-118)

```
Met Ser Asp Leu Thr Asp Ile Gln Glu Asp Ile Thr Arg His Glu Gln
                5                  10                  15
Gln Leu Ile Val Ala Arg Gln Lys Leu Lys Asp Ala Glu Arg Ala Val
            20                  25                  30
Glu Val Asp Pro Asp Asp Val Asn Lys Asn Thr Leu Gln Ala Arg Gln
        35                  40                  45
Gln Thr Val Ser Ala Leu Glu Asp Lys Leu Ala Asp Tyr Lys Arg Arg
    50                  55                  60
Met Ala Asp Ala Val Ser Arg Lys Lys Met Asp Thr Lys Pro Thr Asp
65                  70                  75                  80
Pro Thr Gly Ile Glu Pro Asp Asp His Leu Lys Glu Arg Ser Ser Leu
                85                  90                  95
Arg Tyr Gly Asn Val Leu Asp Val Asn Ala Ile Asp Ile Glu Glu Pro
            100                 105                 110
Ser Gly Gln Thr Ala Asp
        115
```

FIG. 5

rN 1a(1-79)

```
Met Ser Asp Leu Thr Asp Ile Gln Glu Asp Ile Thr Arg His Glu Gln
                  5                  10                  15
Gln Leu Ile Val Ala Arg Gln Lys Leu Lys Asp Ala Glu Arg Ala Val
             20                  25                  30
Glu Val Asp Pro Asp Asp Val Asn Lys Asn Thr Leu Gln Ala Arg Gln
         35                  40                  45
Gln Thr Val Ser Ala Leu Glu Asp Lys Leu Ala Asp Tyr Lys Arg Arg
     50                  55                  60
Met Ala Asp Ala Val Ser Arg Lys Lys Met Asp Thr Lys Pro Thr
 65                  70                  75
```

FIG. 6

Peptide 4 (241-270)

Glu Lys Glu Cys Pro Phe Ile Lys Pro Glu Val Lys Pro Gly Thr Pro
5 10 15
Ala Gln Glu Ile Glu Met Leu Lys Arg Asn Lys Ile Tyr Phe
20 25 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/00180

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/12, C07K 14/175
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Arch Virol, Volume 130, 1993, K. Yoshimatsu et al, "Protective immunity of Hantaan virus nucleocapsid and envelope protein studied using baculovirus-expressed protein", page 365 - page 376, See Table 1 and Discussion --	1-3
X	Journal of Medical Virology, Volume 46, 1995, Olli Vapalahti et al, "Human B-Cell Epitopes of Puumala Virus Nucleocapsid Protein, the Major Antigen in Early Serological Response", page 293 - page 303, page 297 - page 298 and figure 4 --	1-3

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search	Date of mailing of the international search report
21 May 1997	25 -05- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/00180

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of General Virology, Volume 73, 1992, Olli Vapalahti et al, "Cloning and sequencing of Puumala virus Sotkamo strain S and M RNA segments: evidence for strain variation in hantaviruses and exprression of the nucleocapsid protein" page 829 - page 838 ---	1-3
X	WO 9506250 A1 (UNIVERSITY OF NEW MEXICO), 2 March 1995 (02.03.95), See pages 12-13, 21,22, page 25, line 5 - line 16, page 30, line 16 - page 32, line 5, page 34, line 15 - page 35, line 12 and claims 41 and 52 --- --------	1

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Information on patent family members

02/04/97

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