T-helper and humoral responses to Puumala hantavirus nucleocapsid protein: identification of T-helper epitopes in a mouse model

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Puumala hantavirus (PUUV) is a rodent-borne agent causing nephropathia epidemica in humans, a milder form of haemorrhagic fever with renal syndrome occurring in Fennoscandia, central Europe and western Russia. In this study we characterized the immunogenicity of an E. coli-expressed nucleocapsid (N) protein of PUUV (strain Kazan-E6) in inbred mice (BALB/c, CBA and C57/BL6). The recombinant N (rN) protein raised PUUV-specific antibodies in all three tested murine haplotypes, and all IgG subclasses were detected. Epitope mapping using peptides spanning the N protein revealed that the B-cell recognition sites were mainly located at the amino-terminal part of the protein. Proliferative T-helper (Th) lymphocyte responses were detected in all haplotypes after a single immunization with rN. Several Th-recognition sites, spanning amino acids 6–27, 96–117, 211–232 and 256–277, were identified using overlapping peptides. Peptides representing the identified sites could also prime Th-lymphocytes to proliferate in response to recall with rN protein, thereby confirming the authenticity of the identified sites. The rN-primed Th-lymphocytes produced predominantly interleukin (IL)-2 and gamma interferon, together with lower levels of IL-4 and IL-6, indicating a mixed Th1/Th2 response.

Introduction

Puumala virus (PUUV) is a member of the genus Hantavirus, which includes viruses causing different forms of haemorrhagic fever with renal syndrome (HFRS). The clinical symptoms of HFRS are characterized by fever, thrombocytopenia, renal failure and, in severe cases, haemorrhage caused by capillary leak syndrome (Lundkvist & Niklasson, 1994; Hjelle et al., 1995; Kanerva et al., 1998). PUUV occurs in Scandinavia, Finland, western Russia and central Europe and causes a mild form of HFRS named nephropathia epidemica (NE) (Plyusnin et al., 2000). The more severe forms of HFRS are caused by Hantaan (HTNV) (Asia) and Dobrava (Europe) viruses, while Seoul virus (SEOV) (worldwide) causes an intermediate form (Lee et al., 1978; Antoniadis et al., 1996; Lundkvist et al., 1997a). Hantaviruses are carried by specific rodent hosts [the bank vole (Clethrionomys glareolus) for PUUV] and virus transmission from rodents to humans is thought to occur via inhalation of aerosolized animal excreta (Brummer-Korvenkontio et al., 1980; Plyusnin et al., 1996). Approximately 200 000 cases of HFRS occur annually and the mortality varies between < 0·1 and 10%, depending on the causative virus (Lundkvist & Niklasson, 1994; Plyusnin et al., 1996). Another group of hantaviruses, including Sin Nombre (SNV) and Andes viruses found in North and South America, causes hantavirus pulmonary syndrome (HPS) (Nichol et al., 1993; Lopez et al., 1996). This disease is characterized by acute respiratory distress and has a mortality rate of approximately 50%.

Hantaviruses are enveloped and have a three-segment negative-stranded RNA genome packed in helical nucleocapsids. The genome encodes four structural proteins: the L-segment encodes the RNA polymerase, the M-segment the two envelope glycoproteins (G1 and G2) and the S-segment encodes the nucleocapsid (N) protein (Plyusnin et al., 1996; Schmaljohn, 1996).

The role of the immune response in protection, as well as in the pathogenesis of hantavirus infection, is not clear. Hantaviruses causing both HFRS and HPS infect human endothelial cells without any apparent cytopathic effect (Pensiero et al.,...
Production of recombinant nucleocapsid protein. The open reading frame (ORF) of the PUUV (strain Kazan-6) N protein gene was cloned and sequenced by Lundkvist et al. (1997b). The N ORF, encoding amino acids (aa) 1–433, was amplified from cDNA with primers 5′-TTG CAT GCT TAT GAG TGA CTT GAC AGA CAT CCA A 3′ and 5′-TTG TCG ACT TAA TCA TAT CTT TAA GGG CTC CTG 3′, containing a SphiI and a Sali restriction site, respectively. The N ORF was cloned into the pQE-32 vector (Qiagen), containing a T5 promoter and encoding a histidin tag to facilitate purification, according to the manufacturer’s instructions. Competent M15[pREP4] E. coli cells (Qiagen) were transformed and spread on Luria–Bertani agar containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. Selected colonies were grown in super broth medium containing antibiotics, as above, and induced with IPTG (1 mM). Induced mini cultures were screened for expression of N protein by immunoblotting with a pool of PUUV N-specific monoclonal antibodies (MAbs) (1C12, 4C3 and 5B5), as previously described (Lundkvist et al., 1991). Colonies containing a fusion protein of the expected size were selected for further amplification and purification. The rN protein was extracted and purified, using the histidin tag, on a column containing nickel–agarose (QIAGEN), provided by the manufacturer and expressed in the same system was used as a control. The purified protein was dialysed against PBS (Slide-A-Lyser 10,000 MWCO; Pierce), and the protein concentration was determined by measuring the absorbance at 280 nm. The purity and size was determined by Coomassie Blue staining of standard SDS–PAGE gels and immunoblotting as above or with a MAb specific for the histidin tag (Tetra His MAb; Qiagen).

The sequence of the cloned S gene was confirmed by nucleotide sequence analysis using sequencing primers provided by the vector supplier (Qiagen). Cycle sequencing was carried out on plasmid DNA as described previously (de Carvalho Nicacio et al., 2000a).

Synthetic peptides. Eighty-four overlapping peptides (17 aa with 12 aa overlaps) spanning the N protein sequence of PUUV (strain Kazan-6, aa 1–433) were synthesized by multiple peptide synthesis (Syro MultiSyn Tech; Syntex) Using Fmoc-protected amino acids as previously described (Arikawa et al., 1997). The peptides were cleaved and deprotected according to standard protocols for Fmoc peptide synthesis.

Mice. Inbred mice of three different haplotypes, H-2d (BALB/c), H-2b (C57/B16) and H-2k (CBA), were used for analyses of rN immunogenicity. All mice were inoculated at 4–8 weeks of age and were obtained from BK Universal (Solventuna, Sweden).

Immunizations. Determination of antibody responses was carried out on sera from groups of five to six mice immunized intraperitoneally with 20 µg of rN protein emulsified in Freund’s complete adjuvant (FCA). The mice were boosted 4 weeks later subcutaneously with 50 µg of rN protein emulsified in Freund’s incomplete adjuvant and sera were collected by retroorbital bleedings at 2, 4 and 6 weeks.

For proliferation assays and cytokine detection, groups of mice were immunized subcutaneously in the base of the tail with 50 µg of rN protein or control protein emulsified in FCA. For peptide immunizations, mice were injected with 100 µg of peptide emulsified in FCA.

ELISA. Total IgG responses to PUUV N were measured by ELISA essentially as described previously (Hörling et al., 1996). Briefly, rabbit anti-PUUV serum diluted 1:400 in 0.05 M bicarbonate buffer, pH 9.6, was adsorbed to micortitre plates overnight at 4°C. After blocking of non-saturated binding sites with 3% BS A, native PUUV antigen diluted
T-helper and humoral responses to PUU virus

**Fig. 1.** Analysis of the *E. coli*-expressed rN protein (lanes 1, 4), and DHFR control protein (lanes 2, 5) and native PUUV antigen (lanes 3, 6) by immunoblot analysis of transferred proteins detected with a pool of PUUV N-specific MAb (lanes 1–3) and a polyhistidine-specific MAb (lanes 4–6). The migration of molecular mass standards is indicated in kDa on the left.

**Fig. 2.** (a)–(c) Humoral responses to PUUV rN protein immunization in mice. Mice were immunized with rN protein and given a booster dose at 4 weeks. Pools of sera (*n* = 6), were tested in duplicate at 4-fold dilutions starting at 1:250. Antibody responses at (a) 2, (b) 4 and (c) 6 weeks after the first immunization of H-2d (■), H-2k (E) and H-2b (▲) mice. (d)–(f) IgG subclass distribution in (d) H-2d, (e) H-2k and (f) H-2b mice after immunization with PUUV rN protein at 2 (●) and 6 (■) weeks after the first immunization. Each value represents the reciprocal endpoint titre of pools of sera. Cut-off values for positive samples were set at *A* = 0.1.

In dilution buffer (0.5% BSA and 0.05% Tween 20 in PBS) was incubated for 1 h at 37 °C. Serum dilutions were incubated for 1 h at 37 °C, and specific antibody binding was detected with alkaline phosphatase (ALP)-conjugated donkey anti-mouse IgG antibodies diluted 1:5000 (Jackson Immunoresearch), followed by incubation with *p*-nitrophenyl phosphate substrate (Sigma). IgG subclass responses were detected with goat anti-mouse IgG1, IgG2a, IgG2b or IgG3 antibodies diluted 1:5000, followed by incubation with an ALP-conjugated rabbit anti-goat IgG diluted 1:5000 (Sigma) and substrate as above.

- **Identification of B-cell recognition sites.** Linear B cell epitopes were mapped by PEPSCAN (Geysen et al., 1987). In total, 80 peptides (10 aa with 5 aa overlaps) spanning the N protein sequence of PUUV
Fig. 3. Epitope scan analysis of antibody reactivity to peptides spanning the PUUV N protein. The first bar corresponds to aa 1–10, the second bar to aa 6–15 etc. Each bar indicates the antibody reactivity of serum pools from PUUV rN protein-immunized mice: (a) H-2d, (b) H-2k and (c) H-2b. The reactivity of serum pools from non-immunized mice was subtracted.

For determination of cytokine concentrations in in vitro restimulated cell cultures, supernatants were harvested at 24 h for measurement of IL-2 and at 48 h for IL-4, IL-6 and IFN-γ. Cytokine concentrations were measured by ELISA according to the manufacturer’s instructions (Endogen, Cambridge, MC, USA).

Results

Expression and antigenicity of recombinant PUUV N protein

The expressed rN protein gave a band of the expected size (about 54 kDa) by immunoblot with a pool of PUUV-specific MAbs or a polyhistidine-specific MAb (Fig. 1). Additional protein bands with apparent molecular masses of approximately 30, 25 and 20 kDa were visualized together with N, suggesting that they represent truncated forms of N. The control protein, DHFR, gave a band of the expected size (about 26 kDa) by immunoblot with the polyhistidine-specific MAb. The rN protein was also characterized with a panel of 8 N-
specific MAbs which recognized all the epitopes earlier seen in E. coli-expressed polyHis-tagged PUUV rN proteins (Elgh et al., 1996; Lundkvist et al., 1996; Vapalahti et al., 1996).

Influence of MHC on humoral responder status

The immunogenicity of rN was analysed in mice of three different haplotypes (H-2^b, H-2^d and H-2^k). The mice were immunized at weeks 0 and 4, and bled every 2 weeks for 6 weeks. The rN protein was found to be highly immunogenic in mice; 4 weeks after the first injection all tested haplotypes had specific antibody titres of more than 10000. The H-2^k haplotype was the strongest responder, whereas the H-2^d, and in particular the H-2^b haplotype, developed weaker responses, when measured against native PUUV antigen in ELISA (Fig. 2a–c).

All IgG subclasses were detected in serum samples from the three haplotypes, drawn 2 weeks after the first and second immunizations, except for IgG3 which was only detected at low levels in the H-2^b haplotype (Fig. 2d–f). After the first immunization, IgG1 was predominant in the H-2^d and H-2^k haplotypes. This IgG1 dominance remained after the second immunization; however, somewhat higher IgG2a titres could be detected in comparison to IgG2b in these two haplotypes. In the H-2^b haplotype, IgG1 and IgG2b dominated followed by IgG2a after one immunization. After the second immunization there were no titre differences between IgG1, IgG2a and IgG2b in the H-2^b haplotype. Taken together, the IgG subclass distribution suggests a mixed Th1/Th2-like response against rN.
Table 1. PUUV nucleocapsid protein peptides identified by Th-lymphocytes

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Amino acids</th>
<th>Peptide sequence</th>
</tr>
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<tbody>
<tr>
<td>2–3</td>
<td>6–27</td>
<td>DIQEEITREHQQLVVARQKLKD</td>
</tr>
<tr>
<td>20–21</td>
<td>96–117</td>
<td>LRYGNLVDVNAIDIEEPSGQTA</td>
</tr>
<tr>
<td>43–44</td>
<td>211–232</td>
<td>QVRNIMSPVMGVIGFSSFVWD</td>
</tr>
<tr>
<td>52–53</td>
<td>256–277</td>
<td>PAQIEEFLKRRVYFMTRQDV</td>
</tr>
</tbody>
</table>

Fine specificity of the humoral responses

Three pools of sera from groups (n = 6) of H-2b, H-2d and H-2k mice, drawn 2 weeks after the second immunization, were analysed for reactivities against antigenic regions within the PUUV N protein. Mapping was performed with PEPSCAN against 86 overlapping decapeptides corresponding to the whole N sequence. The three serum pools all displayed similar reactivity patterns against the peptides (Fig. 3 a–c), and several antigenic regions were detected. The highest reactivity was seen against aa 1–120, showing that the major antigenic region of the protein is located within the amino-terminal part. Some reactivity could also be seen against the central part of the protein, namely aa 166–180, 221–245, 281–290 and 296–305. Only the H-2k serum pool reacted against peptides within the carboxy-terminal part of the protein, representing aa 396–405 and 411–420.

Th-lymphocyte responses to PUUV N

The immunogenicity of the rN protein was further evaluated at the Th-cell level with a proliferation assay in which rN-primed mouse lymphocytes were restimulated in vitro with homologous or control protein. Efficient Th-lymphocyte priming was achieved in all three haplotypes after a single immunization with 50 µg rN protein in FCA (Fig. 4). Consistent with the humoral response, the H-2k haplotype showed the most pronounced proliferative response and the H-2b haplotype the lowest. Up to 25-fold differences in the amount of antigen needed for equal levels of proliferation was seen between the H-2d and the H-2b haplotypes. The rN protein recalled specific Th-lymphocyte proliferation down to antigen concentrations of 32 ng/ml. All three haplotypes responded with equally low proliferation after recall with the control protein, compared to the rN protein restimulation.

The cytokine profile of the polyclonal rN protein-specific Th-lymphocyte response was evaluated. Lymph node lymphocytes primed with rN were analysed with respect to recall cytokine profiles. Th-lymphocytes from all three haplotypes produced predominantly IL-2 and IFN-γ upon recall with rN protein (Fig. 5 a–d). In addition, IL-6 was detected in the culture...
supernatant of H-2^b Th-lymphocytes while lower levels were seen in supernatants from H-2^d and H-2^k mice. Very low concentrations of IL-4 were detected in supernatants of all haplotypes. This indicated that a mixture of Th1/Th2-like PUUV N protein-specific Th-lymphocytes were primed by immunization with rN protein.

**Mapping of Th-lymphocyte epitopes within PUUV N**

Th-lymphocyte epitopes were mapped within the PUUV N protein in proliferation assays utilizing peptides as recall antigens. Lymphocytes from rN protein-primed H-2^d mice were restimulated with 84 overlapping 17-mer peptides (10 µg/ml) spanning the whole PUUV N protein. Four different antigenic regions within four pairs of overlapping peptides were detected (Fig. 6a–b, Table 1). In one proliferation experiment, the highest reactivity was detected against peptides 2–3, 20–21 and 52–53, corresponding to aa 6–27, 96–117 and 256–277 (Fig. 6a). In a second experiment, the highest reactivity was again found against peptides 2–3 and 20–21. In addition, high reactivity was detected against peptides 43–44, corresponding to aa 211–232 (Fig. 6b).

To verify the authenticity of the identified epitopes, we investigated whether the corresponding peptides could prime an immune response against PUUV N protein. Mice of the H-2^d haplotype were immunized with 100 µg of peptide in FCA and 9–11 days later lymphocytes from draining LNs were restimulated *in vitro* with either homologous peptides, heterologous peptides or rN protein. All peptides were able to prime the immune response giving rise to high proliferative responses after recall with rN protein and homologous peptides (Fig. 7a–d). Recall with heterologous peptides did not give rise to proliferation exceeding that in the medium control (data not shown). For maximum proliferation in response to the different recall antigens, up to 30 times higher concentrations of peptides compared to rN protein were needed. However, in the case of recall with peptides 52–53 (aa 256–277) maximum proliferative responses were detected with equal amounts of peptide and recombinant protein. Within each peptide pair, similar proliferation levels were detected after recall with each peptide, with the exception of peptides 43 (aa 211–227) and 44 (aa 216–232) against which a much lower proliferation was detected after recall with peptide 44, indicating that the major part of the epitope is located within peptide 43.

**Discussion**

In this study, we have analysed the PUUV N protein-specific humoral and cellular immune responses in mice after immunization with rN protein.
Based on the in vitro neutralizing activity of G1- and G2-specific MAbs (Dantas et al., 1986; Arikawa et al., 1989, 1992; Lundkvist & Niklasson, 1992), and on passive transfer experiments (Zhang et al., 1989; Schmaljohn et al., 1990; Å. Lundkvist and others, unpublished) the envelope glycoproteins have been assumed to be the main inducers of protective humoral responses. The role of cell-mediated immunity in hantavirus infection has not been studied in detail. However, it has been reported that the cellular response is important both in protection from and in the pathology of hantavirus infections. Virus-specific T-lymphocytes in passive transfer studies in vitro, and both human and mice CTLs in in vitro studies, have been demonstrated to have protective and antiviral activity against hantaviruses (Asada et al., 1987, 1988, 1989; Yoshimatsu et al., 1993; Ennis et al., 1997; van Epps et al., 1999).

The importance of the N protein in induction of immunity to hantaviruses is still not clear. N-specific MAbs have been shown to partially protect mice from HTNV infection and bank voles from PUUV infection (Yoshimatsu et al., 1993; Lundkvist and others, unpublished). Accordingly, the humoral response to the N protein may, in addition to the glycoprotein-specific response, be of importance for immunity, e.g. via antibody-dependent cell-mediated cytotoxicity and/or complement-mediated cytolyisis, since the N protein has not been shown to induce neutralizing antibodies. However, the N protein has also been implicated in cellular immunity in hantavirus infection. In challenge experiments, baculovirus-expressed N protein has been shown to confer complete protection against HTNV in hamsters, without inducing any neutralizing antibodies (Schmaljohn et al., 1990). Also, the amino-terminal aa 1–118 of N protein were shown to be sufficient to give complete protection against PUUV challenge (Lundkvist et al., 1996).

Mapping of the IgG responses by PEPSSCAN detected epitopes throughout the N protein; however, the majority of the detected reactivities were found in the amino-terminal part of the protein. This agrees with earlier studies, in which sera from PUUV-infected bank voles showed the highest activity against the amino-terminal 1–120 aa (Lundkvist et al., 1996). In contrast, the human IgG responses in NE patient sera have been shown to be broader, with reactivity against peptides spanning the whole N protein (Vapalahti et al., 1995; Lundkvist et al., 1995), while the human IgA responses in NE patient sera were shown to be mainly directed to the carboxy-terminal part of the protein (de Carvalho Nicacio et al., 2000b). It should be noted that conformational antibody epitopes, several of which have been located at the amino-terminal part of the PUUV N protein by MAbs (Lundkvist et al., 1991, 1995, 1996), could not be investigated by the methods used in the present study.

Immune responses after rN immunization varied with mouse haplotype. The highest responder was the H-2^k haplotype (CBA), while the H-2^d haplotype (BALB/c) was intermediate and the H-2^b haplotype (C57/Bl6) was the lowest responder. Earlier studies have shown that certain HLA restriction elements can be associated with course of disease during hantavirus infection. Mustonen et al. (1998) reported an increased frequency of a more severe course of NE in patients with HLA B8 and DRB1*0301 alleles, while HLA B27 on the other hand was associated with mild disease. The differences in responder status observed in the present study were of course not indicators of disease severity as the mouse model used is not an infection or disease model. But the responder status in these mice varied when both humoral and cellular responses were studied, indicating that haplotype plays an important role in immune responses against PUUV N protein.

Inflammatory cytokines have been suggested to play important roles in the pathogenesis of hantavirus infection. Several studies have detected elevated levels of IFN-γ, TNF-α and IL-6 on lymphocytes and in sera from HFRS and HPS patients (Huang et al., 1994; Ennis et al., 1997; Krakauer et al., 1994; Linderholm et al., 1996; Mori et al., 1999). Increased expression of TNF-α, TNF-β and platelet-derived growth factor has also been detected in kidney biopsies from NE patients (Tennon et al., 1996). In the present study we analysed the cytokine profile of in vitro rN-restimulated lymphocytes. These cells produced high concentrations of IFN-γ and IL-2, and lower concentrations of IL-6 and IL-4, indicating that the PUUV N protein predominately induced Th1 type cytokines, but also Th2 cytokines in this system.

In mice the IgG subclass distribution is known to correlate with cytokine profile. IgG1 production is mainly promoted by Th2 cytokines, and in contrast, IgG2a production is promoted by Th1 cytokines (Stevens et al., 1988). The subclass distribution after rN immunization seen in this study did not show a clear predominance of either IgG1 or IgG2a after boost injection, suggesting a mixed Th1/Th2 response, concordant with the cytokine response.

Four T-cell recognition sites were mapped within the N protein by using peptides. Two of the regions were located in the highly immunogenic amino-terminal part of the protein, aa 6–27 and 96–117 (Lundkvist et al., 1996). The other two regions were located in the highly variable central part of N, aa 211–232 and 256–277. The authenticity of these regions was confirmed by recall of peptide-primed lymphocytes with rN protein in vitro. This is the first study in which proliferative T-lymphocyte responses to PUU hantavirus have been mapped. Two earlier studies have identified CD8+ and CD4+ CTL epitopes on the N protein of SNV and HTNV (Ennis et al., 1997; van Epps et al., 1999). In the study by van Epps et al., a human CD8+ CTL epitope in the HTNV N protein was identified, aa 12–20, corresponding to the first region detected in the present study (aa 6–27). This poses the question of cross-reactivity and whether this specific epitope/region is an important T-cell epitope among all hantaviruses. When van Epps et al. compared the cross-reactivity of CTLs against target cells pulsed with peptides representing sequences from different hantaviruses, only the HTNV and the SEOV peptides,
which differ only at aa 12, resulted in cross-reactive lysis of target cells. These results may seem discouraging, but as the same epitope could be important for both CTL and Th-lymphocytes, the restricted activation of CTLs may not be applicable for Th-lymphocytes, which possibly have a broader activation pattern leading to T-cell help via cytokine excretion for antibody production and CTL activation. Furthermore, all regions detected in the present study have earlier been shown to react with antibodies in human NE sera (Vapalahti et al., 1995; Lundkvist et al., 1995). In addition, the two regions in the central part of N (aa 211–232 and 256–277) have also been shown to react with sera from experimentally and naturally infected bank voles, the natural reservoir of PUUV (Lundkvist et al., 1996).

In conclusion, T-cell reactive regions on PUUV N protein have been identified using mouse lymph node lymphocytes. Further studies are needed to define the specific epitopes and the role of these epitopes in the immune responses against other hantaviruses and their role in protection against virus infection.

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References


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