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Characterization of cross-reactive and serotype-specific epitopes on the nucleocapsid proteins of hantaviruses

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Abstract

The hantavirus nucleocapsid (N) protein fulfills several key roles in virus replication and assembly and is the major antigen in humoral immune responses in humans and mice. Here we report on epitopes involved in serotype-specific and cross-reactive recognition of the N proteins of hantaviruses using monoclonal antibodies (mAbs) against the N proteins of Andes virus (ANDV) and Sin Nombre virus (SNV). The mAbs define at least twelve different epitopic patterns which span eight sequences, including amino acids 17–59, 66–78, 79–91, 157–169, 222–234, 244–263, 274–286 and 326–338 on the SNV and ANDV N proteins. Studies on the cross-reactivity of these mAbs with different hantavirus N proteins indicated that epitopes located within amino acids 244–286 are related to serotype specificity. We analyzed further the location of epitopes with available three-dimensional structure information including the N-terminal coiled–coil and derived exposed and hidden residues of these epitopes. The generated recombinant N proteins and the characterized mAbs are functional tools being now available for hantavirus diagnostics and replication studies.

Keywords: Hantavirus nucleocapsid; Serotype-specific B-cell epitopes; Monoclonal antibodies; Diagnostics; Structure

1. Introduction

Hantaviruses form enveloped, spherical virions of 80–140 nm in size and have been first isolated in 1978 (Lee et al., 1978). As members of the *Bunyaviridae* family they have a tripartite ssRNA(−) genome coding for a RNA-dependent RNA polymerase, two glycoproteins which are inserted into the viral envelope membrane, and the N protein associated with the viral genome. In humans, the disease caused by hantaviruses has been associated with hemorrhagic fever and renal syndrome (HFRS) in Europe and Asia and with hantavirus cardiopulmonary syndrome (HCPS) in the Americas, where it causes mortality rates up to 50% (Nichol et al., 1993; López et al., 1996). Since the discovery of HCPS viruses in 1993, several hantavirus species have been identified and isolated as New World hantaviruses being ANDV and SNV representative in South America and in North America, respectively. Efforts have been made to characterize prevalent hantavirus strains in America (Johnson et al., 1999; Levis et al., 1998; López et al., 1997; Padula et al., 2000; Spiropoulou et al., 1994), but in most countries more detailed analysis remain to be completed.

To study hantavirus infection in humans and mice, immunological assays can be used which detect either viral antigens or the presence of hantavirus reactive antibodies. The first method owns the advantage that it can detect infections before immune responses arise whilst the latter affords reliable results even when infection has been cleared. Among the structural proteins of hantaviruses, the N protein is known to induce the major humoral immune responses during the acute-phase and convalescent phase of infection (Kallio-Kokko et al., 2001; Zöller et al., 1989). Applying a pepscan approach it has been reported (Lundkvist et al., 1995; Tischler et al., 2005; Vapalahti et al., 1995) that epitopes recognized by sera of infected individu-

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als are dispersed over the entire N protein. In contrast, the use of truncated N protein segments indicated the presence of linear epitopes predominantly within the N-terminal of the N protein (Elgh et al., 1996; Gött et al., 1997; Jenison et al., 1994; Yamada et al., 1995). The discrepancy of results obtained with synthetic peptides compared with data using recombinant truncated proteins has been associated with the possibility that linear epitopes might be hidden or not properly presented in *E. coli*-expressed proteins (Lundkvist et al., 1996; Tischler et al., 2005).

Being an internal component of virions, the N protein suffers less selective pressures from immune responses than the envelope glycoproteins. This is consistent with the finding that the N protein of hantaviruses is the most conserved structural protein (Plyusnin et al., 1996). Further it is known that the N protein is the most abundant viral protein during infection (Elliott et al., 2000). Combining the characteristics of being a highly immunogenic, conserved and abundant antigen, recombinant N proteins are highly suited as diagnostic agents in immunoassays and their usefulness therefore has been well documented (Gött et al., 1991; Lundkvist et al., 1993; Kallio-Kokko et al., 1993; Zöller et al., 1989). However, antibody responses against the N protein induced by one hantavirus cross-react with N proteins of other hantaviruses (Wang et al., 1993). Epitopes involved in cross-reactivity have been related with the 49 N-terminal amino acids (Araki et al., 2001; Jenison et al., 1994). On the other hand, the existence of serotype-specific epitopes on N proteins of Old World hantaviruses has been recognized for a long time (Ruo et al., 1991; Lundkvist and Niklasson, 1992), although knowledge about their exact location yet is still limited to one study (Yoshimatsu et al., 1996) in which the epitope of a Hantaan virus (HTNV) recognized by a single mAb has been mapped within a region spanning amino acids 205–290. Recently, a single chain Fv antibody showing exclusive recognition of SNV N protein has proven the existence of serotype-specific epitopes on the N protein of a hantavirus from America (Velappan et al., 2007).

With the aim to characterize serotype-specific epitopes and to differentiate them from cross-reactive epitopes on N proteins of New World hantaviruses, in the present work we generated a panel of mAbs against recombinant N proteins of ANDV and SNV and identified their antigenic determinates combining partial enzymatic digestion and pepscan analysis. We show that the epitopes are distributed over the entire N proteins and were able to associate some of them with serotype specificity. We further discuss the location of these epitopes with regard to available 3D molecular structures.

2. Materials and methods

2.1. Virus and cells

Vero E6 cells infected with isolate CHI-7913 (Galeno et al., 2002), species Andes virus, genus *Hantavirus*, family *Bunyaviridae* were provided by Dr. Hector Galeno and Dr. Eugenio Ramirez from the Instituto de Salud Pública de, Chile.

2.2. Patient sera

Serum samples from Chilean patients with confirmed HCPS ($n = 29$) were provided by the Instituto de Salud Pública de Chile. Human sera from healthy individuals used as negative controls were obtained from the Hospital Salvador of Santiago de Chile.

2.3. Peptides

Peptides of 42 and 20 amino acids of length corresponding to amino acids 17–59 and 244–263 of the ANDV N protein strain CHI-7913 (Tischler et al., 2003) were synthesized (New England Peptides). The sequences are as follows: amino acids 17–59 QLVTARQKLKDAEKAVEVDPDDVNKSTLQRRAAVSTLETKLG and amino acids 244–263 CPFLPKPKVASEAFMSTNKM.

2.4. Cloning, expression and purification of the ANDV N protein coding region

The coding region of the N protein was isolated from a previously cloned cDNA of ANDV strain CHI-7913 (Tischler et al., 2003) by PCR using specific primers 5′-AGCGTCGACCCATGGCTGCGAAAGCTGGAATGAGCA-3′ forward and 5′-TCAACGCGTGCGGCCGCTTACAACTTGTGTGGCTCTTG-3′ reverse. The expression vector pET32a (Novagen) was used which directs the synthesis of polyhistidine thioreductase fusion proteins. For gene expression, *E. coli* BL21(DE3) cells transformed with the pET32a/N expression vector were used as described elsewhere (Wilhelm et al., 2006). The recombinant N protein containing the polyhistidine tag was solubilized in 5 M urea and purified using nickel nitriloacetate agarose (Qiagen) according to the manufacturer's instructions. The purity and size of the recombinant protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-thioredoxine mAbs (BiosChile I.G.S.A.).

2.5. mAb production

mAbs against the recombinant ANDV N thioredoxin fusion protein, recombinant SNV N protein (kindly provided by Dr. Sergio Pichuantes from Chiron Blood Testing, who also provided the recombinant Puumala virus (PUUV) N protein) and against ANDV peptides spanning amino acids 17–59 and 244–263 conjugated to hemocianine of the mollusk *Concholepas Concholepas* (Blue Carrier, Biosonda) were prepared using the protocol established by Köhler and Milstein (1975). Hybridoma culture supernatants were screened for the presence of N protein-specific antibodies by ELISA and reactive hybridomas cloned by the limiting dilution method.

2.6. ELISA

Recombinant proteins or peptides conjugated to BSA were adsorbed to microtiter plate wells (Polysorb, Nunc) using 100 ng per well. First antibodies consisted in hybridoma culture super-

natants (dilutions of 1:1 to 1:100) or immune serum samples (dilutions of 1:500). Immune sera were pre-incubated with *E. coli* BL21(DE3) extract to absorb *E. coli*-specific antibodies that may be present in the serum samples. The secondary antibodies consisted in anti-mouse immunoglobulin G (H + L) horseradish peroxidase (HRP) conjugate (Sigma) and anti-human polyvalent immunoglobulins (G, A, and M) HRP conjugate (Sigma) which were used in dilutions of 1:5000 and 1:2000, respectively. Tetramethylbenzidine (KPL) was used as substrate and optical density (OD) measured at 450 nm. The average of the ELISA results were classified as absence of reactivity (OD <0.15), moderate reactivity (OD 0.5–1.5) and intense reactivity (OD >1.5).

2.7. Western blot and dot blot.

Recombinant proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes as described by Towbin et al. (1979). Alternatively, 100 ng of recombinant proteins or peptides were applied as dot blots to nitrocellulose membranes and air-dried. First antibodies consisted in hybridoma supernatants (dilutions of 1:1 to 1:100) or immune sera (dilutions of 1:100). As second antibodies anti-mouse immunoglobulin G (H + L)-specific HRP conjugate (Sigma) and anti-human polyvalent immunoglobulins (G, A, and M) HRP conjugate (Sigma) were used in dilutions of 1:5000 to 1:2000, respectively and incubated for 1 h at 22 °C. Staining was performed using 4-chloro-1-naphtol (Pierce).

2.8. Immunofluorescence assay (IFA)

Vero E6 cells infected with ANDV strain CHI-7913 (Galeno et al., 2002) were added onto 10-spot object glasses, air-dried and fixed with acetone. Hybridoma supernatants were applied in dilutions 1:1 to 1:20 and anti-mouse immunoglobulin Fc-specific FITC conjugate (Sigma) was added in a 1:100 dilution and incubations performed at 37 °C in humid chambers. Fluorescence of ANDV infected cells was classified as absence of reactivity, moderate reactivity, and intense reactivity.

2.9. Protein digestion with endoproteinase lysine-C

To achieve a partial digestion of the recombinant SNV N protein, the optimal conditions of digestion time and endoproteinase lysine-C concentration were first studied. The digestion fragments were analyzed through Tricine-SDS-PAGE (Schägger and von Jagow, 1987). Best results were obtained when SNV N protein was digested for 2 h at 37 °C with lysine-C (Böhringer Mannheim GmbH) in a relation of lysine-C:SNV N (1:260 w/w). Finally, the fragments were subjected to Western blot analysis with mAbs directed against SNV N protein.

2.10. Pepscan

In total, 36 13-mer peptides comprising the N sequence of ANDV strain CHI-7913 (Tischler et al., 2003; GenBank accession number AAO86636) and 36 13-mer peptides spanning the N sequence of SNV strain NM H10 (Spiropoulou et al., 1994; GenBank accession number NC_005216) were synthesized (Sigma–Genosys) covalently linked to nitrocellulose membranes (Geysen et al., 1987). These membranes were analyzed for their reactivity with mAbs as previously described (Tischler et al., 2005). Briefly, membranes were blocked with 5% non-fat milk followed by incubation with hybridoma culture supernatants in dilutions of 1:10 to 1:100 for 3 h at 22 °C. Secondary antibodies anti-mouse immunoglobulin G (H + L)-specific HRP conjugate (Sigma) were used in dilutions of 1:5000 and incubated for 1 h at 22 °C. Bound antibodies were detected using the chemiluminescence substrate SuperSignal Ultra (Pierce) and reactivity measured by exposing the membranes 10–60 s to X-ray films.

3. Results

3.1. Synthesis of recombinant ANDV N protein and antigenicity

The recombinant N protein containing the fusion tag (65 kDa) was solubilized and purified as indicated in Section 2.4 (Fig. 1a). To determine the antigenicity of the recombinant ANDV N protein, its reactivity with immune sera of Chilean HCPS patients was assessed. ANDV N reacted in Western blots with all tested immune serum samples ($n = 5$) (data not shown) and all 29 HCPS patient sera recognized the recombinant protein in ELISA (Fig. 1b). Of the tested normal sera ($n = 98$) all but one were negative against the recombinant ANDV N protein (Fig. 1b). Since this serum did not react with other *E. coli*-expressed recombinant proteins and given that some normal serum samples are from donors of the south of Chile were ANDV is prevalent, we believe that the one positive serum might belong to a healthy donor with a resolved past infection. Based on these results we used this recombinant protein for the development of mAbs against the N protein of ANDV.

3.2. Characterization of mAbs against recombinant ANDV N protein

In total, a panel of six hybridomas was obtained against the recombinant N protein of ANDV (see Table 1). All hybridomas recognized ANDV as determined in IFAs of infected Vero E6 cells (data not shown). In Western blots the mAbs were examined for the recognition of linear epitopes. In this assay, all but one (9C1/C12) hybridoma supernatants reacted with the denaturated ANDV N protein (Supplemental Fig. 1a). To examine the specificity of the obtained antibodies, their reactivity with recombinant SNV and PUUV N proteins was examined in ELISA. Of the six hybridoma supernatants, four cross-reacted with the SNV N protein and only one with the more distantly related PUUV N protein (2B6) which conserve 93% and 85% of similarity, respectively with the ANDV N protein (Table 1).

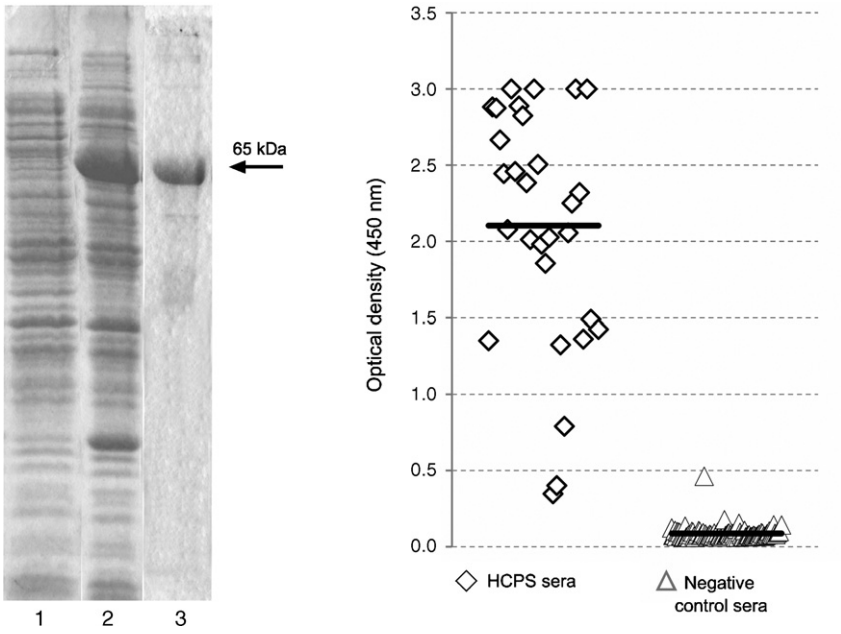

Fig. 1. Synthesis of ANDV N protein in *E. coli* BL21(DE3) and reactivity with patient sera. (a) SDS-PAGE of the ANDV N protein fused to thioredoxine. Lanes 1–3: time 0 after induction with IPTG (1), 3 h after induction with IPTG (2), and purified N protein solubilized in 5 M urea (3). (b) Reactivity of HCPS sera ($n = 29$) and negative control sera ($n = 98$) with the recombinant ANDV N protein in ELISA indicated with rhombuses and triangles, respectively. Black lines indicate the average reactivity of the two sera populations.

To study the epitopes recognized by the antibodies against ANDV N, assays with 36 13-mer peptides covering the whole sequence of the ANDV N protein were performed. A visible example of this experiment can be seen in Fig. 2. In total, two linear epitopes could be identified; the mAb 8F3/F8 reacted with amino acids 274–286 whilst mAb 2B6 recognized amino acids 79–91 (see Table 1). The residual hybridoma supernatants showed no reactivity with linear peptides, although three recognized the recombinant ANDV N protein in Western blots. Probably their epitopes are not properly represented by the non-overlapping 13-mer peptides. In summary, from pepscan and cross-reactivity results it can be concluded that the obtained hybridomas recognize at least four different epitopic patterns (see patterns A–D in Table 1). Further, an exclusive recognition of the ANDV N protein was demonstrated with mAbs 9C1/C12 and 8F3/F8, the latter of which recognizes amino acids 274–286.

3.3. Characterization of mAbs against recombinant SNV N protein

A recombinant SNV N protein, highly reactive with immune sera of HCPS patients ($n = 23$) in dot blot assays (data not shown), was also used for the generation of mAbs. A panel of 11 hybridomas was generated against this protein, of which all but one (1C9/D8) reacted with the SNV N protein in Western blots (Supplemental Fig. 1b). Of these mAbs, all but two (mAbs 3G8/H9 and 2C9/D8) cross-reacted to varying extend with the ANDV N protein in ELISA (Table 2). mAb 3G8/H9 showed a low reactivity with ANDV N and null reaction by Western blot, suggesting that this antibody has a very low reactivity with the non-denaturated form of ANDV N. Cross-reactivity with the PUUV N protein could be detected in ELISA with 5 of the 11 hybridomas (Table 2). This result is consistent with the finding that the PUUV N protein conserves 84% of similarity with the

Table 1
Characterization of antibodies against ANDV N protein

Anti-ANDV N	Western blot ANDV N	ELISA SNV N	ELISA PUUV N	IFA ANDV infected Vero E6 cells	Epitopic pattern[a]	Pepscan ANDV N peptides (amino acids)
1A8/F6	++	++	–	+	A	–
9C1/C12	–	–	–	++	B	–
8F3/F8	+	–	–	+	C	274–286
1A11	++	++	–	++	A	
2B6	++	++	++	+	D	79–91
9B6	+	++	–	++	A	–
7E5/C9	+	+	+	+	E	17–59
8B8/E7	+	–	–	+	F	244–263

"–" indicates absence of reactivity, "+" indicates moderate reactivity, "++" indicates intense reactivity.
[a] Epitopic patterns were organized in concordance to Western blots results with ANDV N, cross-reactivity with SNV N and PUUV N and pepscan results.

Table 2
Characterization of mAbs against recombinant SNV N protein

Anti-SNV N	Western blot SNV N	ELISA ANDV N	ELISA PUUV N	IFA ANDV infected Vero E6 cells	Epitopic pattern of SNV N LysC digestion	Pepscan SNV N peptides (amino acids)
7B3/F7	++	++	++	++	A	17–59
8C10/H2	++	++	–	+	B1	n.d.
2D2/G4	++	++	–	+	B1	n.d.
3F6/C7	++	++	+	+	B3	–
7F1/H9	++	++	–	+	B1	n.d.
3G8/H9[a]	++	+/–	–	–	B2	222–234; 326–338
4F1/D6	++	++	++	+	C	n.d.
5F1/F7	++	++	++	++	C	66–78
7A2/D5	++	++	++	–	D	157–169
1G2/F11	++	+	–	–	E	–
2C9/D8	–	–	–	–	n.d.	n.d.

"–" indicates absence of reactivity, "+" indicates moderate reactivity, "++" indicates intense reactivity.
[a] This mAb reacted considerably less than the others with ANDV N by ELISA and showed no reactivity with ANDV N by Western blot.

SNV N protein. Of the nine supernatants that recognized the recombinant ANDV N protein in ELISA, seven showed reactivity with ANDV in IFAs of ANDV infected Vero E6 cells (data not shown). In the case of mAbs 3G8/H9 and 2C9/D8 against the SNV N protein, the absent reactivity with ANDV in IFAs is in line with the observation that they have a very low or absent reactivity with the ANDV N protein in ELISA. In the future, these mAbs should be examined for their reactivity with cells infected with SNV.

To determine the antigenic regions recognized by the 11 hybridomas against the SNV N protein, we assayed them against the SNV N protein partially digested with lysine-C. Based on the recognition of the different fragments obtained from the partially digested SNV N protein, the mAbs could be organized into five different epitopic patterns (patterns A–E) (Fig. 3). Epitopic pattern A showed reactivity with fragment sizes bigger than 18 kDa whilst epitopic pattern B included in addition the recognition of a fragment of 14 kDa. Epitopic pattern C showed additional reactivity with fragments of 16 and 20 kDa. Epitopic patterns D and E, with one member each, recognized different fragments (see Fig. 3). To identify the epitopic sequences of these epitopic patterns, at least one mAb of each pattern was subjected to peptide scanning studies with 36 13-mer peptides bound to nitrocellulose membranes covering the entire SNV N protein and complementarily with two synthetic peptides spanning amino acids 17–59 and 244–263 (see Table 2). mAb 7B3/F7 of epitopic pattern A reacted with the synthetic peptide spanning amino acids 17–59; mAb 3F6/C7 of epitopic pattern B did not react with any peptide whilst mAb 3G8/H9 of the same epitopic group reacted with two peptides comprising residues 222–234 and 326–338; mAb 5F1/F7 of epitopic pattern C recognized amino acids 66–78 (Fig. 2); mAb 7A2/D5 representing the epitopic pattern D reacted with amino acids 157–169 and mAb 1G2/F11 of epitopic pattern E did not react with any peptide. Within epitopic pattern B mAbs 3G8/H9 and 3F6/C7 showed different cross-reactivity compared with other mAbs of this group (Table 2). These are labeled B2 and B3, respectively. mAb 3G8/H9 is the only member of epitopic pattern B that showed almost no cross-reactivity with the ANDV N protein. Furthermore it recognized two non continuous peptides within the sequence, most probably due to the recognition of a conformational epitope constituted by at least the two identified linear sequences. In contrast, mAb 3F6/C7 is the only member of epitopic pattern B that cross-reacted with the PUUV N protein. Since it did not react with any peptide in the pepscan assay, it probably recognizes another conformational epitope or alternatively, its epitope was not properly represented by the non-overlapping 13-mer peptides.

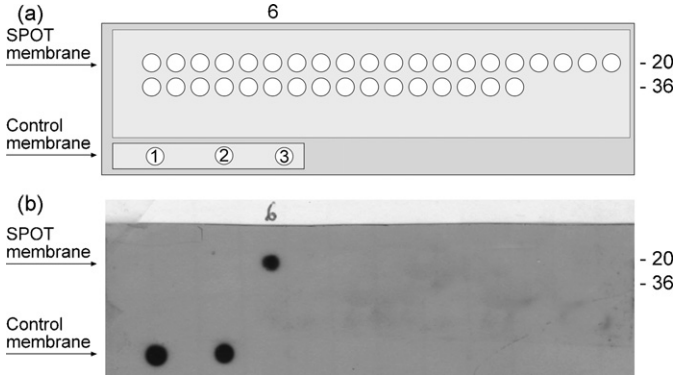

Fig. 2. Visual example of pepscan analysis. (a) Scheme of the used SPOT and control membranes. The SPOT membrane includes 36 13-mer peptides covering the complete N protein of SNV in a non-overlapping manner, 20 peptides in each row. The control membrane includes dot blots with 0.5 μg of ANDV N (1), SNV N (2), and negative control protein (3). (b) Reactivity of the mAb 5F1/F7 anti-SNV N with the SPOT and control membranes. A chemiluminescence substrate was used to visualize antibody binding. The reactive dot on the SPOT membrane corresponds to peptide no. 6 comprising amino acids 66–78 of the SNV N protein. When the SPOT peptides comprising the ANDV or SNV N proteins were incubated with a negative control mAb, no reactivity was observed.

3.4. Characterization of mAbs against peptides representing antigenic regions of the ANDV N protein

Given that the linear immunodominant epitopes in human B-cell responses have been mapped to two principal regions on hantavirus N proteins comprising amino acids 17–59 and 244–263 (Gött et al., 1997; Jenison et al., 1994; Tischler et al., 2005; Vapalahti et al., 1995), mAbs were prepared against peptides spanning these regions within the ANDV N protein.

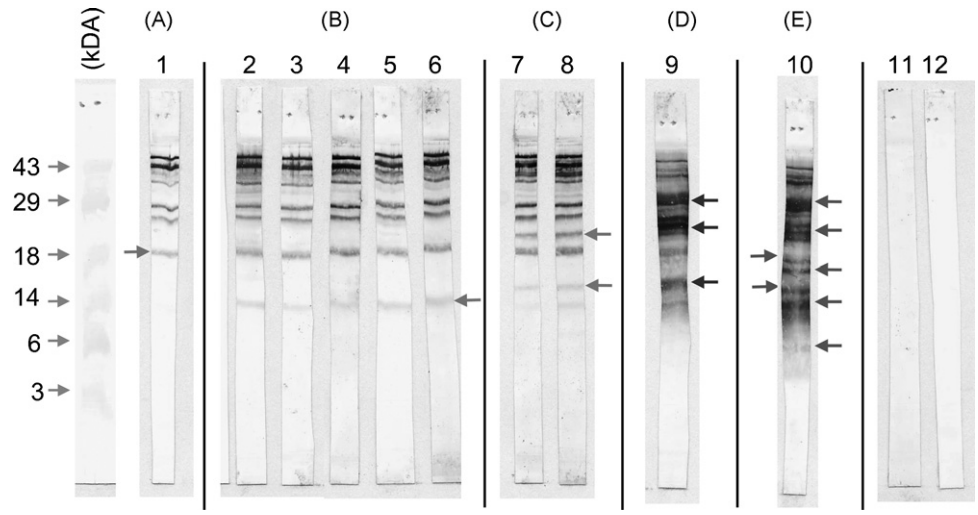

Fig. 3. Epitopic patterns of mAbs anti-SNV N protein. Western blot of SNV N protein digested partially with lysine-C. Lanes 1–12: reactivity of mAbs (1), 3F6/C7 (2), 3G8/H9 (3), 2D2/G4 (4), 7F1/H9 (5), 8C10/H2 (6), 4F1/D6 (7), 5F1/F7 (8), 7A2/D5 (9), 1G2/F11(10), negative control mAb (11), and secondary antibody anti-mouse immunoglobulin G conjugated to HRP (12).

A panel of 10 hybridomas against the peptide comprising amino acids 17–59 was obtained. Of these, only one hybridoma (7E5/C9) reacted with the recombinant ANDV N protein in ELISA as well as in Western blots and cross-reacted with the recombinant SNV N and PUUV N proteins in ELISA (Table 1, epitopic pattern E). This mAb also reacted with ANDV in IFAs of infected Vero E6 cells (Table 1). Additionally a panel of seven hybridomas against the peptide spanning amino acids 244–263 was obtained, of which four recognized ANDV infected Vero E6 cells (8B8/E7, 1E3, 1G6, and 1H5). Consistent with the low sequence conservation of this region, only one hybridoma (1H5) cross-reacted with the SNV N protein and none with the PUUV N protein (data not shown).

4. Discussion

In the present work, we describe mAbs against the N protein of ANDV and SNV and characterize their antigenic determinants. In total, 37 hybridomas were obtained against the N proteins which reacted at least with twelve different epitopic patterns (Tables 1 and 2). Of these mAbs, three showed exclusive recognition of ANDV N and two were specific for SNV N. Given the high sequence conservation among these proteins (93% similarity), the mAbs represent precise tools for discrimination among these hantavirus species. On the other hand, six hybridomas cross-reacted with the N protein of PUUV (85% similarity with ANDV N; 84% similarity with SNV N) and are thus highly suited for a more universal hantavirus detection.

Since antibodies recognize mostly surface structures of the native proteins, the identification of epitopes provides valuable comprehension about the surface topology and antigenicity of proteins (Davies et al., 1990). In this sense, the conservation of antigenic regions among different hantavirus species can be a measure for structural conservation. As seen in Fig. 4, all but one of the epitopes sequences described here coincide with epitopes previously described on N proteins of hantaviruses (Elgh et al., 1996; Gött et al., 1997; Jenison et al., 1994; Lundkvist et al., 1996; Tischler et al., 2005; Vapalahti et al., 1995; Yamada et al., 1995). A new epitope not previously defined on hantavirus N proteins was identified with mAb 3G8/H9 anti-SNV N (amino acids 222–234). In the pepscan approach this mAb reacted with two peptides that are distant in the primary sequence of the protein and do not have significant sequence similarities (^{222}VIGFSFFVKDWME^{234} and ^{326}ELGAFFAILQDMR^{338}). This suggests that these two peptides may be located close to each other on the 3D structure of the native N protein generating a conformational epitope.

Among hantavirus N proteins, the most variable regions are located between amino acids 50–80 and 230–310 (Kaukinen et al., 2005). Therefore, epitopes contained in these regions are more likely to be specific for each hantavirus species. Our results show that none of the antibodies that recognize epitopes located within the N-terminal variable region (7B3/F7 and 5F1/F7) are able to distinguish the two New Word hantaviruses. In contrast, mAb 8F3/F8 and anti-ANDV N peptide 244–263 antibodies that recognize the epitopes comprised of amino acids 274–286 and 244–263, respectively, discriminate among the closely related ANDV and SNV. These epitopes coincide with the C-terminal variable region of the N proteins. Sequence similarities among ANDV and SNV N proteins within these epitopes amount to 73% (amino acids 244–263) and 81% (amino acids 274–286), compared with an overall conservation among these proteins of 93%. Therefore, the non-conserved residues P250, V252, A253, S254, F257, M263, Q278, and I281 on the ANDV N protein may be crucial for serotype-specific antibody binding (compare with Fig. 4). On the other hand, the absence of cross-reactivity of the mAb 3G8/H9 anti-SNV N with ANDV N in Western blot and low cross-reactivity in ELISA assays may be explained by the non-conserved residues of its epitope including S226 and G233 on the SNV N protein.

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ANDV_CHI-7913      17 VTARQKLKDAEKAVEVDPDDVNKSTLQNRRAAVSTLETKLGELKRQL 63
SNV_NM_H10         17 VTTRQKLKDAERAVELDPDDVNKSTLQSRRAAVSALETKLGELKREL 63
PUUV_Sotkamo       17 IVARQKLKDAERAVEVDPDDVNKNTLQARQQTVSALEDKLADYKRRM 63

ANDV_CHI-7913      64 ADLVAAQKLATKPVDPTGLEPDDHLKEKSSL 94
SNV_NM_H10         64 ADLIAAQKLASKPVDPTGIEPDDHLKEKSSL 94
PUUV_Sotkamo       64 ADAVSRKKMDTKPTDPTGIEPDDHLKERSSL 94

ANDV_CHI-7913     151 DNKGTRIRFKDDSSFEEVNGIRKP 174
SNV_NM_H10        151 ENKGTRIRFKDDSSYEEVNGIRKP 174
PUUV_Sotkamo      151 ENKGTRIRFKDDTSFEDINGIRRP 174

ANDV_CHI-7913     209 QVKARNIISPVMGVIGFGFFVKDWMDRIEEF 239
SNV_NM_H10        209 QVKARNIISPVMGVIGFSFFVKDWMERIDEF 239
PUUV_Sotkamo      209 QIQVRNIMSPVMGVIGFSFFVKDWSERIREF 239

ANDV_CHI-7913     244 CPFLPKPKVASE-----AFMSTNKMYFLNRQRQVNESKVQDIIDLIDHAETESATLF 295
SNV_NM_H10        244 CPFLPEQKDPRD-----AALATNRAYFITRQMQVDESKVSDIEDLIADARAESATIF 295
PUUV_Sotkamo      244 CPFIKPEVKPGTPAQEIEMLKRNKIYFMQRQDVLDKNHVADIDKLIDYAASGDPTSP 300

ANDV_CHI-7913     321 VAGVPELGAFFSILQDMRNTIMA 343
SNV_NM_H10        321 VAGMPELGAFFGILQDMRNTIMA 343
PUUV_Sotkamo      326 VAGMAELGAFFSILQDMRNTIMA 348
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Fig. 4. Comparison of epitopes on the N proteins of ANDV, SNV, and PUUV recognized by mAbs and immune sera. The epitopes recognized by mAbs on the ANDV and SNV N proteins are indicated within the respective sequences in red, bold letters. Epitopes recognized by immune sera of humans and rodents on ANDV N (Tischler et al., 2005), SNV N (Jenison et al., 1994; Yamada et al., 1995), and PUUV N (Lundkvist et al., 1995; Vapalahti et al., 1995) are indicated within the respective sequences with underlined letters. In the case of overlapping epitopes identified in different studies, the sequences are indicated in addition with bold, cursive letters.

During the last years, efforts have been made to describe the hantavirus N protein oligomerization as well as its molecular structure. Using three-dimensional reconstruction of images obtained by electron microscopy of negative stained recombinant N protein it has been shown that the N protein oligomerizes into trimers with monomers attached to each other at both ends forming a curved structure (Kaukinen et al., 2004). Based on recent crystallographic data of the N-terminal of the SNV N protein (Boudko et al., 2007) and other studies (Alfadhli et al., 2001; Alminaite et al., 2006) it has been shown that the 78 most N-terminal amino acids of N proteins form an antiparallel intramolecular coiled–coil structure when the protein is not arranged into its trimeric conformation. Based on this information, we analyzed the molecular structure of epitopes localized within the N-terminal of the SNV N structure (PDBid: 2IC9). As seen in Fig. 5, the epitope comprised within amino acids 17–59 (mAb 7B3/F7) is located within the loop structure of the intramolecular coiled–coil including parts of the two alpha helices whilst the epitope spanning amino acids 66–78 (mAb 5F1/F7) consists partially of an alpha helical structure (amino acids 66–73). Coiled–coils are characterized by heptad repeats (abcdefg) (n) where the a and d positions form the interface between alpha-helices (Akey et al., 2001). In the case of the N-terminal intramolecular coiled–coil structure of the SNV N protein, hidden residues comprised within epitopes amino acids 17–59 and 66–73 correspond to L18, T21, L25, A28, V32, N40, L44, R47, V51, L54, L58, L68, and N72. In this structure all these residues are buried within the interface and for this reason may not be involved in antibody binding. Given that the N-terminal of the N protein of more distantly related hantaviruses such as HTNV, PUUV, SNV, and ANDV is known to contain epitopes for antibody binding (Gött et al., 1997; Jenison et al., 1994; Lundkvist et al., 1996; Tischler et al., 2005) it can be assumed that parts of the coiled–coil structure are highly accessible even in the trimeric form of N proteins.

In summary, in the present work we describe epitopes distributed over the entire N protein of two hantaviruses causing hantavirus pulmonary syndrome. Our results confirmed previous studies on human and rodent B-cell epitopes mapped with the pepscan method showing the presence of antigenic regions over the entire N protein and not merely at its N-terminal (Lundkvist et al., 1995; Tischler et al., 2005; Vapalahti et al., 1995). Fur-

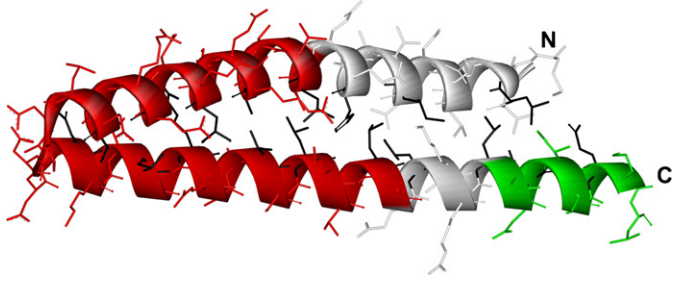

Fig. 5. Localization of SNV N protein epitopes on the coiled–coil domain structure spanning residues 1–75 (PDBid: 2IC9) (Boudko et al., 2007). Ribbon representation of SNV N residues 1–75. Amino acids involved in epitope amino acids 17–59 (mAb 7B3/F7) are indicated in red and the partial epitope amino acids 66–75 (mAb 5F1/F7) are indicated in green. Residues involved in positions a and d of the heptad repeat are indicated in black.

thermore, the present data was used to relate recognized epitopes with their molecular structure within the protein (amino acids 17–59 and 66–78) and allowed us to associate the antigenic regions of amino acids 244–263 and 274–286 with high serotype specificity. The generated recombinant proteins and the mAbs are now available for the development of rapid and sensitive immunoassays as well as for studies on the role of the different epitopic regions in specific functions such as RNA binding and interactions with viral and cellular proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2008.01.013.

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