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Cross-reactive and serospecific epitopes of nucleocapsid proteins of three hantaviruses: Prospects for new diagnostic tools

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ABSTRACT

The diagnosis of infectious diseases is sometimes difficult because of extensive immunological cross-reactivity between related viral antigens. On the path of constructing sero-specific antigens, we have identified residues involved in sero-specific and cross-reactive recognition of the nucleocapsid proteins (NPs) of Puumala virus (PUUV), Seoul virus (SEOV), and Sin Nombre virus (SNV) using serum samples from 17 *Nephropathia epidemica* patients. The mapping was performed by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis on a panel of N protein derivatives and alanine-substitution mutants in the three different hantavirus backgrounds. Four regions with different serological profiles were identified encompassing the amino acids (aa) 14–17, 22–24, 26, and 35–38. One of the regions showed strong cross-reactivity and was important for the recognition of SEOV and SNV antigens, but not the PUUV antigen (aa 35–38). Two regions displayed perceivable SEOV characteristics (aa 14–17 and aa 22–24 and 26) and the combined result of the alanine replacements resulted in a synergetic effect against the PUUV antigen (aa 14–17, 22–24, 26).

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1. Introduction

Viruses of the *Hantavirus* genus in the *Bunyaviridae* family include several of the most serious human pathogens that cause haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). HFRS accounts annually for up to 150,000 diagnosed cases world-wide (Lee and Wang, 1996), and the clinical severity of HFRS ranges from asymptomatic infections to fulminant hemorrhagic shock and death.

Hantaan virus (HTNV) and Dobrava virus (DOBV) are old world viruses. The causative agents of severe forms of HFRS have mortality rates of up to 15% and a large portion of these patients develops haemorrhages (Krüger et al., 2001; Linderholm and Elgh, 2001). In contrast, diseases caused by Puumala virus (PUUV), Seoul virus (SEOV), and Saaremaa virus (SAAV) are less severe (Plyusnin et al., 1999; Schmaljohn and Hjelle, 1997). HCPS caused by Sin Nombre virus (SNV) or Andes virus (ANDV) is prevalent in North and South America, respectively. These viruses are of particular public concern due to the high case-fatality rates (Castillo et al., 2001; Hooper et al., 2001) and the risk for person-to-person transmission reported for ANDV (Enría et al., 1996; Ferres et al., 2007; Martinez et al., 2005). The similarity between different hanta virus proteins is high, but interestingly these small differences seem to be pivotal for the outcome of the disease.

In general, hantaviruses are transmitted to humans by aerosols of rodent excreta, and the different hantaviruses are consequently restricted to the geographic regions inhabited by their specific rodent hosts (Schmaljohn and Hjelle, 1997). Because different rodent habitats overlap and some rodent species are able to host more than one hantavirus serotype, several hantaviruses are found in the same geographical area. In some regions of central Europe, PUUV, DOBV, and SAAV are found (Kallio-Kokko et al., 2005) while in parts of Asia HTNV, SEOV, and PUUV (Kim et al., 1995; Ruo et al., 1994; Zhang et al., 2007) coexist. As many as eight different hantavirus species types have been reported to co-circulate in Russia, and at least four of them are known to infect man (Tkachenko et al., 2007).

Because of the high similarity between different hantavirus proteins, serological cross-reactions are common (Chu et al., 1995; de Carvalho Nicacio et al., 2002; Elgh et al., 1998), and consequently it is difficult to conclude the causative hantavirus agent in areas

Abbreviations: PUUV, Puumala virus; SEOV, Seoul virus; SNV, Sin Nombre virus; HFRS, hemorrhagic fever with renal syndrome; HCPS, hantavirus cardiopulmonary syndrome; NP, nucleocapsid protein; aa, amino acids.

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where multiple hantaviruses coexist. These diagnostic problems, due to high cross-reactivity between related viruses, are not only restricted to the hantavirus genus. Similar difficulties are also found when diagnosing viruses of the Flaviviridae (e.g., Dengue Fever Virus) family by immunological methods (Kao et al., 2005).

The nucleocapsid protein (NP) is the major antigenic protein in all hantaviruses, and PUUV-infected individuals display a strong and early antibody response towards the NP (Kallio-Kokko et al., 2001; Vapalahti et al., 1995). This powerful immune response makes this particular protein suitable for use in diagnostics.

In a previous study, it was shown that mice vaccinated with cDNA encoding the immunogenic hantavirus NPs display high homologous and cross-reactive titers. The reciprocal cross-reactivities were found to be highly individual both in titers and reactivity profiles towards heterologous NPs (Lindkvist et al., 2007).

This study was performed with serum samples of *Nephropathia epidemica* patients as a “one-way” cross-reactivity study towards geographically distinct hantavirus NPs. The locations of the antigenic regions were identified and key residues were pinpointed by using the above serum samples against three sets of NPs, NP derivatives, chimeric NPs, and NP alanine substitution mutants. We found four amino acid regions (epitopes), before and around amino acid 40 near the amino terminus of the hanta virus NPs that contribute to the different homologous and cross-reactive profiles.

The idea of identifying epitopes involved in homologous and/or heterologous reactivities and modifying them to create serotype specific antigens is attractive. We believe that these types of analyses are important on the path to construct more specific diagnostic tools to distinguish between the immunologically related hantavirus infections. This concept of using serotype specific antigens could also be useful in the diagnostics of other infectious diseases with similar serological cross-reactivities.

2. Methods

2.1. Clinical samples

Serum samples were collected from patients hospitalized at Norrlands Universitetssjukhus Sweden with a typical clinical picture of *Nephropathia epidemica* (NE). All patients contracted the disease in the county of Västerbotten, Sweden. The Puumalavirus infections were confirmed serologically in acute-phase and convalescent serum collected on the day of hospitalization and 2–3 months after recovery.

The Research Ethics Committee of Umeå University approved this project.

2.2. Generation of expression plasmids

PCR products of the complete S-genes and gene fragments encoding the nucleocapsid proteins (NP), and NP derivatives of PUUV, SEOV, and SNV (Accession numbers; PUUV Umeå/hu, AY526219; SEOV Sapporo SR-11, M34881; SNV Convict Creek 107, L33683) were amplified and cloned into the prokaryotic expression vectors pTrcHis2 TOPO®TA and pET101/D-TOPO® (Invitrogen™ Life technologies), respectively. Obtained PCR products were ligated in frame with DNA encoding a carboxyl-terminal His-tag for purification and detection of the expressed gene products. Using the QuikChange® Site-Directed Mutagenesis Kit (Stratagen Inc.), according to the instructions of the manufacturer, alanine substitution mutants were constructed. Furthermore, six synthetic genes were synthesised (Eurofins Medigenomics GmbH, Germany), three of them encoding the first 80 amino acids (aa) of the three hantavirus S-genes (PUUV, SEOV and SNV) with the amino acids 14–43 replaced by a corresponding non-related amino acid sequence of the NP of Rift Valley Fever virus (RVFV) (GenBank accession number AF134534). Three additional synthetic genes were constructed to express the first 120 aa of the RVF NP with an insertion of aa 14–43 of PUUV, SEOV or SNV S-genes, respectively. Before use, the DNA constructs (Fig. 1) were all verified by sequencing by MWG-Biotech AG, Germany.

2.3. Antigen purification

All DNA constructs encoding the NPs and NP derivatives of PUUV, SEOV, and SNV, as well as the RVFV NP derivatives containing hanta NP insertions were expressed from poly-histidine-fusion vectors in *Escherichia coli* BL-21 DE3 (Invitrogen™ Life technologies). Briefly, transformed bacteria were grown in LB media (Luria-Bertani) containing 100 μg/ml of carbencillin to an optical density at A_{600} of about 0.6. The expression of the antigens was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) to a final concentration of 0.5 mM. The bacterial cells were harvested by centrifugation at 5000 rpm for 15 min 2–4 h after induction, and the NP antigens were affinity purified under native conditions using metal chelating chromatography according to a slightly modified protocol from QIAexpressionist 01/2000 (Qiagen Ltd., UK).

2.4. Enzyme-linked immunosorbent assay and Western blot

Enzyme-linked immunosorbent assays (ELISA) and Western blot analyses were performed essentially as described before (Lindkvist et al., 2007). Briefly, ELISA microtiter plates were coated with 3 μg/ml of the NP antigens, and after blocking, convalescent sera were added and the plates were incubated over night at 4 °C. Horseradish peroxidase conjugated goat anti-human IgG, A, M secondary antibody (AdB SeroTec, MorphoSys AG) was used, and a minimum of two separate measurements with duplicates of each serum sample and dilution were done. The sera were diluted fourfold until specific reactivity towards the antigens was lost.

The statistical comparisons between titers of each serum sample towards the full length NPs was performed by one-way Analysis of Variance (ANOVA) using Bonferroni post hoc *t*-tests. Comparisons of each mutant titer to respective wild type titers were performed for each serum sample. The titer data were analyzed by ANOVA using Dunnett’s 2-sided post hoc test. For both types of analyses, three levels of significance are indicated: $^{*}p$-value < 0.05; $^{**}p$-value < 0.01; and $^{***}p$-value < 0.001.

For Western blot analyses, the serum samples were diluted 1:1000 and incubated for 1h at room temperature before the secondary antibody was added. Dilutions and reagents were basically the same as in the ELISA-analyses.

3. Results

3.1. Mapping the major epitopes to the amino-terminus of the NPs

Serum samples were collected of NE patients to investigate if Puumala virus-infected humans display similar individual cross-reactivity profiles as previously observed in sera from DNA-vaccinated mice (Lindkvist et al., 2007). In addition, we proceeded and identified the locations of the major epitopes, and some key residues in the antigenic regions of the NPs of PUUV, SEOV, and SNV. For this purpose, we analysed 17 human serum samples using a panel of NP derivatives and mutants constructed and purified from bacterial cultures expressing these different antigens. Since previous data indicated that the amino-terminus is the principal region harbouring dominant B-cell epitopes, the initial experiments became a comparison of the titers between the full-length

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