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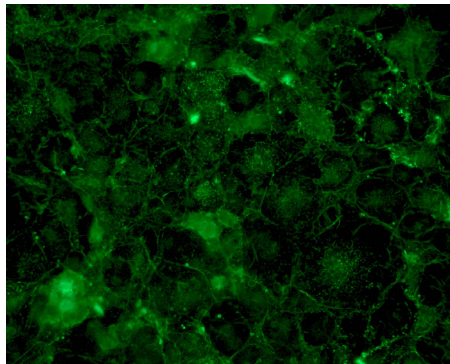

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Creation of synthetic genes in order to increase the cross-reactivity and protective efficacy of Hantavirus DNA-vaccines

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Abstract

Annually, hantaviruses infect more than 150,000 people worldwide, resulting in thousands of deaths, why prophylactic treatment such as vaccination against human pathogens among the hantaviruses is highly desirable. The immunological cross-reactivity and high genetic conservation between these viruses could be one key towards a common vaccination strategy. In this study, we have initiated an investigation exploring the possibility of creating one "universal" DNA-vaccine against several of the human pathogenic hantaviruses. We have constructed two synthetic genes, multi-epitope constructs, derived from proposed B- and T-cell epitopes of the S-genes from five different hantaviruses. The epitopes have been modified for increased cross-reactivity by individual amino acid substitutions towards common consensus sequences within the proposed epitope. Furthermore, the individual epitopes were flanked with immuno-stimulatory DNA sequences (CpG) for enhanced immunogenicity. This report contains the initial results from the efforts of exploring the possibility of a multi-epitope vaccine with an enhanced cross-reactivity. The synthetic genes and the corresponding full-length S-genes were successfully expressed in bacteria for the production of antigens used to evaluate the immune response obtained in animal model systems. The genes were also expressed in eukaryotic cells, indicating the possibility of *in vivo* expression of the encoded antigens in vaccinated animals. Interestingly, the synthetic proteins turned out to be very stable, which is favorable for the generation of a strong and long-lasting immune response.

1. Introduction

One of the greatest medical achievements in history is the discovery and development of vaccines. Diseases that have plagued mankind for centuries are today largely controlled, brought to the brink of extinction and even entirely exterminated through the prophylactic treatment of vaccination. However, there are still many infectious diseases for which no preventive treatment exists. While some pathogens induce weak or no protection at all in the individual after recovery from the natural infection, others induce a massive immune response towards epitopes that do not elicit protection. Another strategy employed by some pathogens is to use components of the immune system of the host to enter and infect new cells (*e.g*. Dengue fever virus). Lethal infections of viral origin, such as HIV have yet withstood all vaccination trials. Therefore, since many traditional methods of vaccination are unsuccessful, new vaccination strategies have to be addressed. Hopefully, modern gene technology provides the necessary means to realize such new ideas.

The Hantavirus genus in the *Bunyaviridae* family of viruses includes several human pathogens that cause severe haemorrhagic fever with renal syndrome (HFRS) on the Eurasian continent or Hantavirus pulmonary syndrome (HPS) in the Americas. [1,2] The severity of the disease greatly depends on the virus in question with a mortality ranging from over 40% for certain New World viruses to less than 1% for others. [1,3,4] In total HFRS, which mainly affects the kidneys, annually accounts for 150,000-200,000 cases worldwide, which results in thousands of deaths. [1] Rodents are the natural host for hantaviruses, and since all members of the genus are carried by specific rodent hosts, the viruses are restricted to the geographical area inhabited by their particular rodent host. Virus transmission to humans occurs through inhalation of aerosols of contaminated excreta such as urine, faeces or saliva from infected rodents. [5] Thus, the risk of acquiring an infection is largely associated with outdoor activities or the presence of rodents in proximity of human lodgings. [3] There are currently four viruses associated with HFRS; Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV) and Puumala virus (PUUV). The latter group of viruses is frequently found in Sweden and causes a mild form of HFRS, *Nephropathia epidemica* (NE). The Sin Nombre virus (SNV) is an example of a virus causing HPS, which has the distinction from HFRS to affect the lungs and leave the kidneys largely unaffected .

Although viruses of the *Bunyaviridae* family are genetically remote, they are similar in many other respects; the virus particles are spherical with a bilipid membrane derived from the host cell[3] and all viruses contain a tripartite negative strand RNA genome [6]contained in separate nucleocapsids within the virus particle. The segments are denoted L (large), M (medium) and S (small) and for the hantavirus genus they encode four proteins: The L-segment encodes the RNA-dependent RNA polymerase, the M-segment contains the genetic information for a poly-protein which is processed into two glycosylated envelope proteins (G_1 and G_2), and the S-segment encodes the multifunctional nucleocapsid protein (N) [7] (fig. 1).

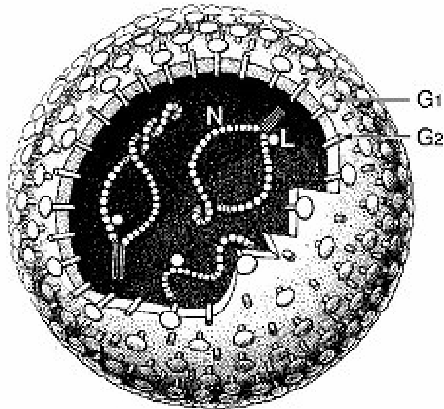

Figure 1. Schematic picture of the Bunyavirus particle. The tripartite negative strand genome (Large, Medium and Small) is protected by oligomerized nucleocapsid (N) protein subunits in separate nucleocapsids. Each part of the genome is associated with an RNA-dependent RNA polymerase (L) and the structural proteins on the virion surface (G_1 and G_2) are encoded by the M-segment.

The PUUV N-protein is a major antigenic determinant, which induces a strong antibody-response in the early phase of the NE-disease. [8,9] Therefore, the N-protein has frequently been used as antigen in vaccination studies. When comparing the amino acid sequences of the N-proteins from the five hantaviruses mentioned above, it becomes apparent that they are highly conserved. The nucleocapsid proteins of HTNV, DOBV and SEOV display an amino acid sequence identity of approximately 80%, whereas when including PUUV and SNV in the comparison, the sequence identity is reduced to about 60% (fig. 2).

Identity (%)

Divergence (%)

	DOB	**HTN**	**SEO**	**SN**	**PUU**
DOB		83.2	80.2	62.1	59.9
HTN	19.1		82.5	62.4	60.6
SEO	23.1	20.0		61.2	61.1
SN	50.7	50.2	52.5		71.0
PUU	55.2	53.8	52.5	34.9	

Figure 2. Divergence/Identity comparison of the hantavirus N-proteins (generated by the MegAlign program in the DNAstar package®).

Due to the genetic similarity between these five different hantaviruses, serological cross-reactivity has been observed in a number of studies [10-13]. Normally, this feature constitutes an

adverse effect of a vaccine, since for the most part the main purpose is to confer immunity against one specific pathogen. However, the concept of this study is rather the reverse. In the case of the human pathogenic hantaviruses, the high cross-reactivity may be the key to a common vaccination strategy against these viruses. The antigenic properties of the N-protein, together with the high degree of conservation and the serological cross-reactivity indicate that a synthetic gene, based on regions of the S-gene, could be used as a universal DNA-vaccine against different Hantavirus serotypes.

Traditional vaccines consist primarily of killed or attenuated pathogens, or peptide/protein subunits thereof. When killed pathogens or subunits are used as vaccines, the humoral arm of the immune system is induced since this part of the immune system primarily acts on extracellular components. In order to induce cellular immunity, attenuated pathogens or related harmless pathogens are used. These pathogens are able to enter the cells of the vaccinee and prime the cellular arm of the immune system. However, these live vaccines can pose unacceptable risks for people with a weakened immune system (elderly people, cancer patients undergoing chemotherapy or persons suffering from AIDS) by their ability to sometimes cause full-blown diseases. Yet another intolerable risk by using attenuated pathogens is the possibility of their reversion to virulent forms, which is one major obstacle against vaccine development regarding pathogens that cause fatal diseases. [14]
In contrast, DNA-vaccines consist of double-stranded DNA that is unable to cause infection. Usually plasmid vectors, containing an inserted gene encoding an antigenic protein from a selected virus or bacterium, are used and the antigen-encoding DNA is introduced to the vaccinee without the virulent capacity of the actual pathogen. [14] There are several advantages of using these types of vaccines. There is a possibility to choose favourable antigens, which is important since some pathogens use strong immunogens as decoys to delude the immune system while other, less prominent structures, might prime a more efficient immunoreaction towards the invasive organism. [15] The cost-effectiveness of large-scale production and the less demanding storage conditions for DNA-vaccines, in comparison to other alternative vaccine formulations, is not to be neglected. [16] The major advantage of using DNA-vaccination, however, is the fact that the antigen is produced inside the cells of the vaccinee, imitating the situation of a natural viral infection. These aspects make the development of genetic vaccines an encouraging field in the search for the ultimate viral prophylactic treatment.

The aim of this study was to construct synthetic genes encoding multiple epitopes modified for maximal cross-reactivity towards the nucleocapsid protein of five different hantaviruses, and to express these genes in pro- and eukaryotic expression systems. Our further objective is to examine the potential of these genes for use as a universal DNA-vaccine protecting against the different Hantavirus serotypes included. The construction strategy was based on the identification of individual conserved B- and T-cell epitopes in the N-proteins followed by modification of these epitopes in order to increase cross-reactivity of the immune-response in a mouse animal model. In addition to the synthetic genes, the full-length S genes were included in the study. The constructs were successfully expressed in bacterial and eukaryotic systems and in future work the immune response will be evaluated after immunizing BALB/c mice with these wt S-genes.

2. Material & methods

Construction of mini-genes

The oligonucleotides (purchased from MWG-Biotech, Germany) used to create each mini-gene were designed to hybridize and form the desired DNA sequence with necessary 5'- and 3'-overhangs corresponding to the restriction enzyme sites used (Table 1, fig. 3).

Table 1. The oligonucleotides used in the construction of the mini-genes.

Construct	Oligo-nucleotide	Sequence (5’-3’)
1A	1	CGCCGGCTGACGTTGGGCCAGCTGGTTATCGCTCGCCAGAAAGTTAAAGACGCTGAAAAACA
1A	2	GTACGAAAAAGACCCAGACGACCTGAACAAACGCGCTCTGACCGACCGCGCTCGAGCTTAAGC
1A	3	TCTGGCGAGCGATAACCAGCTGGCCCAACGTCAGCCGGCGGTAC
1A	4	GTCGTCTGGGTCTTTTTCGTACTGTTTTTCAGCGTCTTTAACTT
1A	5	GGCCGCTTAAGCTCGAGCGCGGTCGGTCAGAGCGCGTTTGTTCAG
1B	1	CGCCGGCTGACGTTGCAGCAGCTGGTTGTTGCTCGCCAGAAACTGAAAGACGCTGAACGCCGCT
1B	2	TCCGCACCATCGTTTGTGGCCTGTTCGAAGACATCAACGGCATCCGCAAACCTCGAGCTTAAGC
1B	3	TTTCTGGCGAGCAACAACCAGCTGCTGCAACGTCAGCCGGCGGTAC
1B	4	GAACAGGCCACAAACGATGGTGCGGAAGCGGCGTTCAGCGTCTTTCAG
1B	5	GGCCGCTTAAGCTCGAGGTTTGCGGATGCCGTTGATGTCTTC
2	1	TCGAGGCCGGCTGACGTTGCTGAGCACCCGCGGCCGCCAGACCGTTAAAGAAAACAAAGGCACCCG
2	2	CTTCCGCACCATCGTTTGTGGCCTGTTCGAAGACGTTAACGGCATCCGCAAAGATCGATTATAAGC
2	3	TTAACGGTCTGGCGGCCGCGGGTGCTCAGCAACGTCAGCCGGCC
2	4	ACAGGCCACAAACGATGGTGCGGAAGCGGGTGCCTTTGTTTTCT
2	5	GGCCGCTTATAATCGATCTTTGCGGATGCCGTTAACGTCTTCGA
2	2Correction3’	CATAACGATGCGGCCGCTTTGCGGATGCCGTTAACGTCTT
3	1	CGATGCCGGCTGACGTTGGACATGCGCAACACCATCATGGCTAGCAAAACCGTTGGCACCAGCGTTT
3	2	ACCTGACCAGCTTCGTTGTTCCAATCAGCATCTGGGTTTTCGCTGGCGCTCCAGGAATTCTGTAAGC
3	3	TTGCTAGCCATGATGGTGTTGCGCATGTCCAACGTCAGCCGGCAT
3	4	ATTGGAACAACGAAGCTGGTCAGGTAAACGCTGGTGCCAACGGTT
3	5	GGCCGCTTACAGAATTCCTGGAGCGCCAGCGAAAACCCAGATGCTG
4	1	AATTCGCCGGCTGACGTTGGAACTGCGCGGCCTGGCTCAGGCTCTGATCGA
4	2	CCAGAAAGTTAAATGTATCTACGTTGCTGGCATGGCTGAACTGGGCGCTTTCGGATCCTAAGC
4	3	ACATTTAACTTTCTGGTCGATCAGAGCCTGAGCCAGGCCGCGCAGTTCCAACGTCAGCCGGCG
4	4	GGCCGCTTAGGATCCGAAAGCGCCCAGTTCAGCCATGCCAGCAACGTAGAT
4	4Correction 5’	ATAGACTGTAGCCGGCGAATTCTGACGTTGAATTCGCCGGCTGACGTTGGA
4	4Correction 3’	CGGAGCGCGGCCGCTTAGGATCCGAAAGCGCCCAG

The annealing procedure was performed in GIBCO/BRL 1×ligation buffer by heating the mixture of phosphorylated oligonucleotides (10 pmol/µl) to 95°C for 10 minutes, and thereafter cooling the mixture slowly to room temperature (r.t.). Each annealed mini-gene mixture was then ligated to a digested, dephosphorylated and gel purified pBluescriptIISK(-) vector. The ligation mixtures were purified by ethanol precipitation and used for transformation of electrocompetent TOP10F’ *E.coli* cells. Electroporation was performed using a genepulser (Bio-Rad Gene Pulser® II, Bio-Rad Laboratories, CA) at 2.45 kV and 25 µF. The DNA purification kits used were QIAGENs QIAprep Spin Miniprep Kit® and JETsorb GelExtraction Kit®. Restriction enzymes were purchased from MBI Fermentas and Roche Applied Science. The transformed cells were plated

on LA-plates containing IPTG (0.5 mM), X-gal (0.01%) and carbencillin (100 μg/ml). As the DNA insertion into the multiple cloning site of the pBluescriptIISK-vector disrupts the α-fragment of the β-galactosidase gene, colonies containing a plasmid with an insert are colourless in contrast to blue colonies, which contain an "empty" vector. The transformants obtained were screened by PCR using one primer annealing to the insert together with either forward or reverse pBluescript primer as a vector primer. Transformants, resulting in PCR products of the expected size when analysed by gel-electrophoresis, were cultured in 1×LB medium (Luria-Bertani, containing 100 μg/ml carbencillin over night (o.n.) and plasmid DNA was prepared using the Qiagen QIAprep Spin Miniprep Kit®. The correct nucleotide sequences of the mini-genes were confirmed by DNA sequencing (MWG-Biotech, Germany). The mini-genes were based on sequences from the nucleocapsid genes of the following genetically related hantaviruses PUUV, DOBV, SEOV, HTNV and SNV (Table 2).

Table 2. Accession numbers of the wild-type virus sequences used in the initial amino acid comparison.

Nucleocapsid genes	Accession number	Reference
Dobrava virus	L41916	Avsic-Zupanc,T., Toney,A., Anderson,K., Chu,Y.K. and Schmaljohn,C. Genetic and antigenic properties of Dobrava virus: a unique member of the Hantavirus genus, family Bunyaviridae. J. Gen. Virol. 76 (Pt 11), 2801-2808 (1995)
Hantaan virus	M14626	Schmaljohn,C.S., Jennings,G.B., Hay,J. and Dalrymple,J.M. Coding strategy of the S genome segment of Hantaan virus. Virology 155 (2), 633-643 (1986)
Seoul virus Sapporo	M34881	Arikawa,J., Lapenotiere,H.F., Iacono-Connors,L., Wang,M.L. and Schmaljohn,C.S. Coding properties of the S and the M genome segments of Sapporo rat virus: comparison to other causative agents of hemorrhagic fever with renal syndrome Virology 176 (1), 114-125 (1990)
Sin Nombre virus (Convict Creek)	L33683	Schmaljohn,A.L., Li,D., Negley,D.L., Bressler,D.S., Turell, M.J., Korch, G.W., Ascher,M.S. and Schmaljohn, C.S. Isolation and initial characterization of a newfound hantavirus from California Virology 206 (2), 963-972 (1995)
Puumala virus Umeå	Manuscript in preparation	Johansson,P., Holmström,A., Olsson,M., Lindgren,L., Juto,P., Elgh,F and Bucht, G. Complete gene sequence of a human Puumala hantavirus isolate, Puumala Umeå; Characterisation of the encoded gene products and a sequence comparison. Manuscript in preparation

Creation and cloning of synthetic genes and wild-type S-genes

The mini-genes were ligated together one by one in the pBluescriptII SK(-) vector to form one single open reading frame encoding an array of different hantavirus epitopes (fig. 4). This procedure was repeated until two variants of the synthetic genes had been created, Syn1A and Syn1B, each consisting of four mini-genes. Furthermore, a sequence encoding an N-terminal signal-peptide, to ensure export of the peptide into the ER, and a sequence encoding a FLAG-epitope enabling detection of the expressed proteins were added to the 5' flanking region of the mini-genes by sub-cloning the two constructs into a modified pUC19 vector[17]. In a second sub-cloning step, the synthetic genes and the regulatory elements mentioned, were transferred to eu-

and prokaryotic expression vectors (pcDNA3.1/V5-His® TOPO©TA and pTrcHis2 TOPO©TA (Invitrogen), respectively). Also, the corresponding wild-type S-genes from the different hantaviruses were cloned in two steps as described above into the eukaryotic and prokaryotic expression vectors. (Table 2).

Prokaryotic expression of synthetic and wt S-genes
The pTrcHis2 TOPO©TA vectors containing synthetic or wt S-genes were transformed into TOP10 chemically competent *E.coli* cells (according to the pTrcHis2 TOPO©TA cloning kit manual from Invitrogen) and plated on selective medium. Transformants were screened by PCR and verified by DNA sequencing as described earlier. Correct clones were inoculated in 1×LB containing 100 µg/ml carbencillin o.n. and the following morning the cultures were diluted 500 times in fresh LB-medium. Protein expression was induced by adding IPTG to a final concentration of 1mM when the OD_{600} of the culture was between 0.5 and 0.7. For each protein, aliquots to be analysed for the optimal induction conditions were removed at intervals during the induction period. Plasmids were also prepared from each culture using QIAGENs QIAprep Spin Miniprep Kit® and the subsequent sequencing was performed by MWG-Biotech, Germany.

Eukaryotic expression of synthetic and wt S-genes
The eukaryotic expression vectors pcDNA3.1/V5-His® TOPO©TA containing synthetic or wt S-genes were transformed into TOP10 chemically competent *E. coli* cells as described earlier and plated on selective medium. Correct transformants were identified by PCR and cultured o.n. in 1×LB containing 100 µg/ml carbencillin. High quality plasmid preparations were performed using Hi-Speed Plasmid Midi Kit™ (Qiagen). The constructs were introduced into COS-1 cells (ATCC CRL 1640) by electroporation using approximately 1.5-2 × 10^6 cells, 20 µg plasmid DNA and a genepulser (Bio-Rad Gene Pulser™, Bio-Rad Laboratories, CA) at settings 200V and 960 µF, according to. [18] The cells were cultivated in Dulbecco's modified Eagle's medium containing Glutamax-I (GIBCO/BRL, Life Technologies Inc., USA) supplemented with 10% fetal calf serum, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml of streptomycin. The cells were grown in a humidified atmosphere in 5% CO_2 at 37ºC to about 90% confluence before harvesting. The cells were maintained for 72 h post-transfection before analysis by Western blot or by immunofluorescence. For the Western blot analysis, adherent cells were washed three times with ice-cold PBS (0.1 M sodium phosphate (pH 7.2) and 0.145 M NaCl) and harvested using a cell scraper. For immunofluorescence, the cells were maintained in BD Falcon CultureSlides™ and fixed in PBS containing 1% Saponin and 4% paraformaldehyde and stained as described by the manufacturer of the specific antibodies used.

Western blot
When analysing eukaryotic protein expression, comparable amounts of COS-1 cells expressing synthetic and wild-type N-protein were harvested as described above, treated in SDS-PAGE sample buffer and separated by electrophoresis. The proteins were transferred to Immobilon™ transfer membranes (Millipore Corporation) and thereafter incubated in a blocking solution composed of 0.5 M NaCl, 0.01 M Tris, 0.05% Tween-20, pH 8.2 (TBS-T) containing 5% non-fat milk. The membranes were incubated for 1h at r.t. with the primary antibody, which was diluted in TBS-T containing 2.5% non-fat milk, according to the manufacturer's recommendations. For the detection of the synthetic proteins the anti-FLAG antibody (Sigma Aldrich, Inc.) was used and for the detection of the hantavirus N-proteins an anti-V5 antibody (USBiological) was used. The membranes were washed 3×10 min in TBS-T before incubation with the secondary antibody; sheep anti-mouse Ig, horseradish peroxidase (Amersham Life Science), for 1h at r.t. The membranes were washed as described before and the detection was performed using ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech) and

Cronex® MD Developer C buffer according to the manufacturer's instructions (Sterling Diagnostic Imaging, Germany).
The Western blot analyses for the detection of the prokaryotically expressed proteins were performed in a similar manner, however, the primary antibody used was an anti-His antibody (Amersham Pharmacia biotech).

Immunofluorescence assay
Expression of the cloned genes in eukaryotic cells was also visualised by immunofluorescence. COS-1 cells were transfected by electroporation and cultivated in 8-well CultureSlides as described earlier. Three days post transfection adherent cells were washed 2×5 min in PBS and subsequently fixed in fixation-solution (PBS containing 1% Saponin and 4% Paraformaldehyde) for 15 min. The fixed cells were then washed 3×5 min in PBS containing 1% Saponin (Saponin-PBS) and incubated for 30 min with a blocking buffer composed of 0.1% blocking reagent (Boehringer-Mannheim) in Saponin-PBS. The primary antibodies (anti-FLAG or anti-V5) were diluted in blocking-buffer (1:250 and 1:200 respectively) and incubated with the cells for 1h. After three washes (5 minutes each) with Saponin-PBS a secondary antibody was added (Alexafluor 488 goat-anti-mouse antibody, Molecular probes). Thereafter, the cells were incubated in complete darkness for 1h before the final washes (3×5 min in Saponin-PBS and 1×5 min in PBS). Finally, anti-fade solution was added (Prolong® anti-fade kit, Molecular probes) and glass slides to cover the cells were applied. Analysis of the cells was performed using a fluorescence microscope (DMR microscope, Leica).

3. Results

Prediction and design of B- / T-cell epitopes and generation of synthetic genes
In this study, an approach to improve the immune response following DNA-vaccination is presented. The intention was to create synthetic genes derived from linear epitopes of the highly antigenic nucleocapsid proteins of the five genetically related hantaviruses PUUV, DOBV, SEOV, HTNV and SNV (Table 2). As the concept was based not only on the selection of specific epitopes, but also on the idea of epitopes being adjusted to increase cross-reactivity, the selected epitopes were modified for this purpose. Linear B-cell epitopes in the nucleocapsid proteins of hantaviruses has been suggested in several publications[3,6,7,19], and the most immunogenic epitopes were chosen. The corresponding sequences in the five different viruses were compared at protein-level for sequence similarity, and five different B-cell epitopes were selected on the basis of conservation and reported immunogenicity (Table 3). Consensus sequences identified by protein alignment, using the MegAlign program/Clustal method in the DNAstar® package, constituted the basis for prediction of the T-cell epitopes. T-cell epitopes restricted to BALB/c mice (haplotype $H2\text{-}K^d$) were identified by using the T-cell epitope prediction program "SYFPEITHI epitope prediction", and by suggestions according to van Epps, H. et al. and Park, J. et al.. [20,21] Several T-cell epitopes with MHC class I and MHC class II restriction were found, and four of each type were chosen and slightly modified for further investigation, (Table 3). Amino acids important for binding to the MHC class I or MHC class II molecules (anchoring residues) were left unchanged.

Table 3. Selected B- and T-cell epitopes. Bold letters in the T-cell epitopes indicate the anchoring residues.

Epitope type	Residue numbers in the S-segment	Amino acid sequence	Virus with the highest degree of similarity
B-cell epitope 1A	16-30	GQLVIARQKVKDAEK	DOB, HTN, SEO
B-cell epitope 1B	16-30	QQLVVARQKLKDAER	PUU, SN
B-cell epitope 2	141-155	LSTRGRQTVKENKGT	PUU, SN
B-cell epitope 3	341-355	DMRNTIMASKTVGTS	DOB, HTN, SEO
B-cell epitope 4	409-423	ELRGLAQALIDQKVK	PUU, DOB
MHC I epitope 1A	31-40	Q**Y**EKDPDD**L**N	DOB, HTN, SEO
MHC I epitope 1B, 2	197-205	R**F**RTIVCG**L**	PUU, SN
MHC I epitope 3	124-133	V**Y**LTSFVV**P**I	DOB, HTN, SEO
MHC I epitope 4	323-331	CI**Y**VAGMAE**L**	PUU, DOB, HTN, SEO, SN
MHC II epitope 1A	39-47	**L**NK**R**A**L**TD**R**	DOB, HTN, SEO, SN
MHC II epitope 1B	165-173	**F**ED**I**N**G**IR**K**	PUU, SN
MHC II epitope 2	165-173	**F**ED**V**N**G**IR**K**	PUU
MHC II epitope 3	308-318	**S**IW**V**F**A**GAP	PUU, DOB
MHC II epitope 4	325-334	YVAG**M**AE**L**G**A**F	PUU, DOB, HTN, SEO, SN

Five mini-genes were designed (fig. 3), and the codons were modified after statistics from codon-usage frequency in BALB/c mice and *E. coli*. Each mini-gene was designed to contain one B-cell epitope, and two T-cell epitopes flanked by a single CpG-motif (5'-GTCGTT-3') as suggested by Hartmann, G. et al [22]for increased immunogenicity and for separation of the different epitopes.

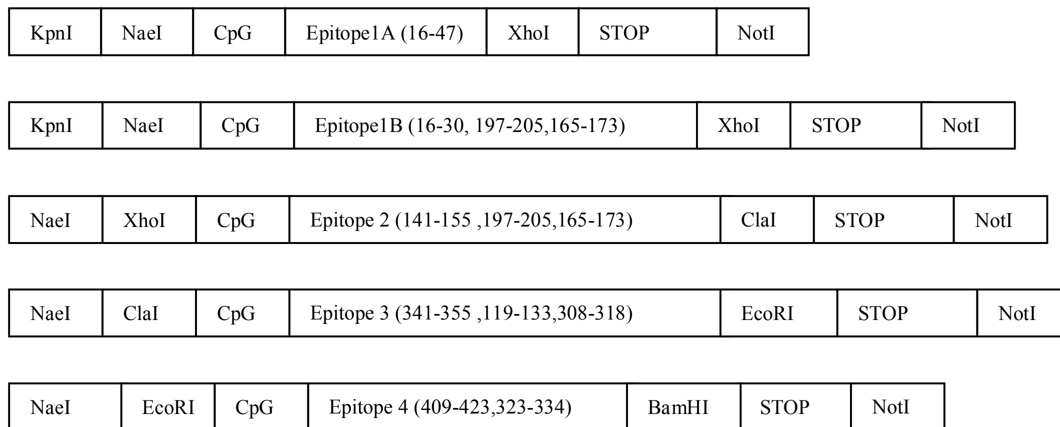

Figure 3. Schematic picture of the mini-genes 1, 1B, 2, 3 and 4. The numbers enclosed by the parenthesis indicate the amino acid positions for the B-, T_{MHCI}- and T_{MHCII}-epitopes in the corresponding genes.

The restriction enzyme sites flanking each mini-gene made it possible to assemble the mini-genes as an ordered array of epitopes (fig. 4). These mini-genes were ligated together using the indicated restriction enzyme cleavage sites and cloned into the prokaryotic vector pBluescript II SK(-).

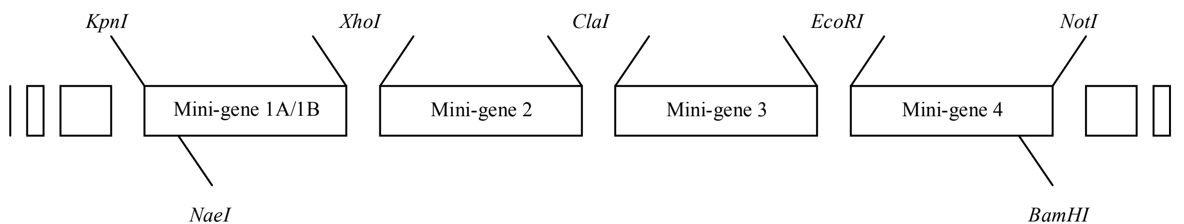

Figure 4. The array of mini-genes in the pBluescript vector. The minigenes 1A/1B – 4 were ligated together using restriction enzyme sites *KpnI/XhoI*, *XhoI/ClaI*, *ClaI/EcoRI* and *EcoRI/NotI* respectively. The synthetic genes were constructed in such a way that each individual mini-gene could be removed and exchanged by using the indicated restriction enzyme cleavage sites.

Expression and analysis of prokaryotic and eukaryotic proteins

The synthetic genes and the corresponding wild-type S-genes were sub-cloned twice (as described in materials and methods), resulting in constructs encoding the proteins of interest fused to the regulatory elements needed for expression and detection in pro- and eukaryotic systems. It is of importance to express and purify (from bacteria) the wild-type and synthetic proteins to use them as antigens in further analyses of immune serum from vaccinated mice. It was also imperative to verify the expression of the corresponding proteins in eukaryotic cells to ensure that the proteins could be expressed in mammalian cells (mimicking post-vaccination expression). The proteins were therefore expressed in bacteria, and before and during protein induction, samples were removed and analysed by Western blot in order to verify protein expression and to determine the protein yield. Interestingly, the synthetic gene products proved to be more stable than the corresponding wild-type N-proteins as the highest yield of synthetic proteins was produced after approximately 20 h compared to 4 h for most of the wt N-proteins

(fig. 5). The synthetic genes were also expressed in transfected COS-1 cells, albeit at a reduced level (fig. 6).

The wild-type N-proteins of SEOV, SNV and PUUV were abundantly expressed in prokaryotic and eukaryotic cells, and when analyzed by Western blot they were found to be of the expected size (fig. 5, 6 and 7). However, the bacterially produced HTN N-protein was rapidly degraded during the time of induction. Therefore, the bacteria producing the HTN N-protein were harvested at a lower OD than the others (the optimal induction time was 20 min), resulting in reduced protein concentration (fig. 5). Since the addition of protease-inhibitors did not inhibit the degradation of the HTN N-protein, this protein was purified in bigger batches than the other proteins. The eukaryotic HTN clone was maintained in the cells for 72 h before harvest, and also in this case only a small fraction of full-length protein was detected (fig. 6). When studying the results of the immunofluorescence analysis of transfected eukaryotic cells, the recombinant HTN N-protein was clearly expressed, but by fewer cells when compared to the N-proteins of SEOV, SNV and PUUV (fig. 7). This indicates that the small amount of HTN N-protein observed is not entirely a result of a lower stability of the protein, but also reflects a reduced transfection-frequency for the HTN-S construct.

The DOBV N-protein was expressed at a satisfactory level, but unfortunately, when expressed in bacteria also this recombinant protein was degraded. Only a minor fraction of the protein produced corresponded to full-length DOBV N, while the major part constitutes degraded peptides (fig. 5). Also in this case, the addition of protease-inhibitors did not inhibit the degradation of the protein, and neither did the use of the protease negative *E. coli* strain BL21-SI improve the protein yield. This problem must be addressed before evaluating the synthetic genes for use in DNA-vaccination. When analysed by Western blot, the eukaryotic expression of the DOBV N-protein was similar to that of the HTNV N-protein, where only a small fraction of full-length protein is detected (fig. 6). And also in this case the immunofluorescence analysis revealed a reduced amount of DOBV N-positive cells, indicative of a reduced transfection-frequency for the DOBV construct as well (fig. 7).

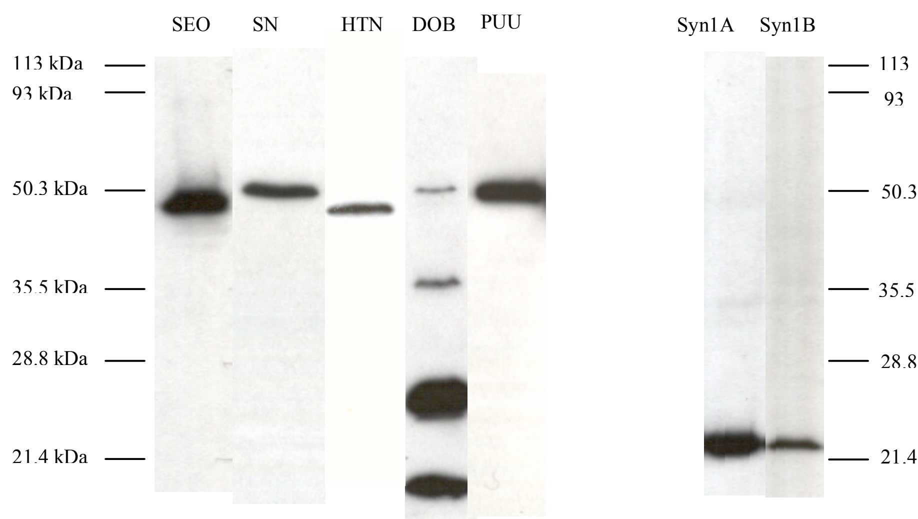

Figure 5. Western blot analysis of prokaryotically expressed wt S- and synthetic genes (Syn1A and Syn1B). The proteins were detected by using an α-His antibody.

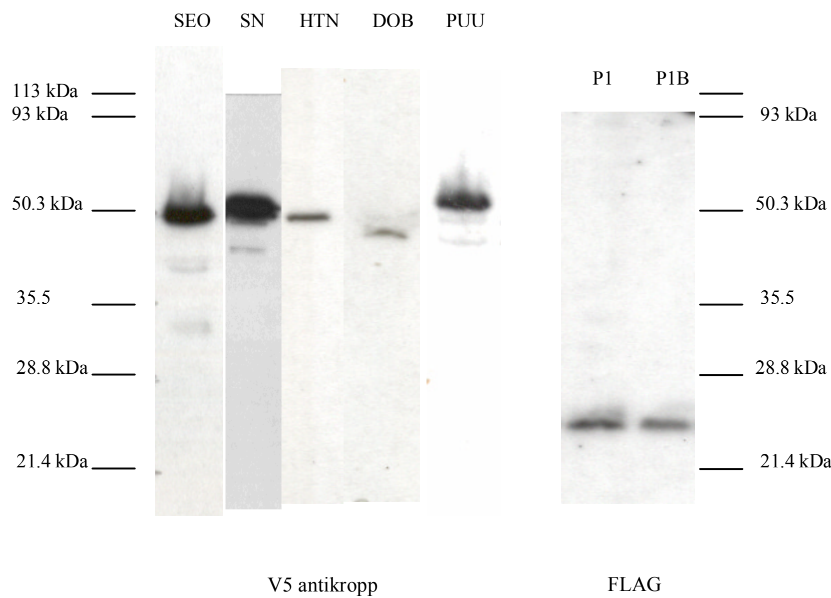

Figure 6. Western blot analysis of total cell extract from transfected COS-1 cells 3-days post-transfection. The expression of the wt N-proteins were detected using an α-V5 antibody. The synthetic proteins were detected using an α-FLAG antibody.

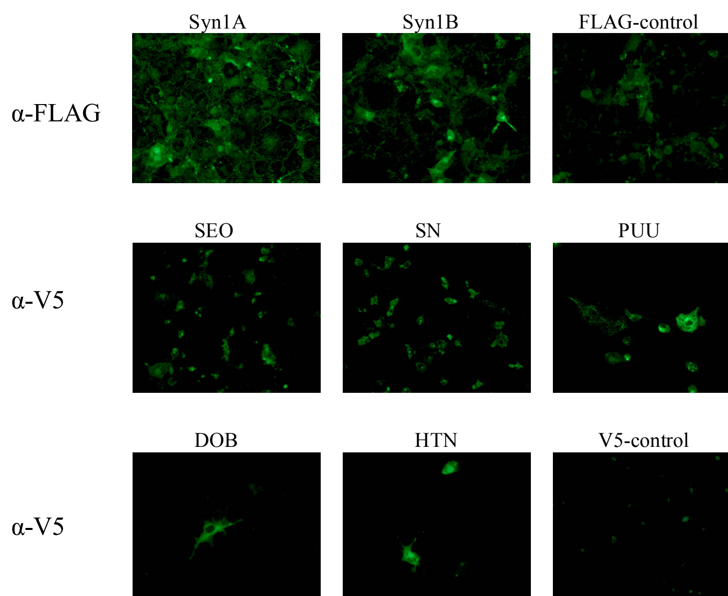

Figure 7. Immunofluorescence analysis of COS-1 cells transfected with synthetic genes and corresponding wt S-genes. From the top left corner; Synthetic gene 1, synthetic gene 1B (using α -FLAG antibody) and a negative control for α -FLAG antibody. Middle row from the left, wt S-genes; SEO, SN and PUU (using α –V5 antibody). From the bottom left corner, wt S-genes; DOB, HTN (using α –V5 antibody) and a negative control for α –V5 antibody.

4. Discussion

In this study, one possible approach to improve the immune response after vaccination is presented. The intention was to create a universal DNA-vaccine against genetically related hantaviruses, where the concept is based not only on the selection of specific epitopes, but also on the idea of epitopes being adjusted to increase cross-reactivity. Only a limited number of studies have been performed regarding the examination of vaccines against human pathogenic hantaviruses. Today, very few commercially available vaccines exist, [23,24] and those are primarily directed against hantavirus infections in Asia. These vaccines are generally manufactured after traditional vaccine formulas, consisting of killed or attenuated virus particles from tissues of infected animals with the risks and limitations accompanying such formulations. Therefore, the cross-reactivity observed between several hantaviruses [10,12,13,23,25,26] together with the increasing use and broad acceptance of multi-epitope constructs in vaccine research [27-31]has served as the fundament for this study.

Synthetic epitopes derived from PUUV, DOBV, SEOV, HTNV and SNV S-genes were created and modified with the purpose of increased cross-reactivity based on the multi-epitope approach and the antigenic properties of the hantavirus nucleocapsid proteins. In this part of the study, the aim was to investigate whether such synthetic genes can be expressed *in vitro*, thereby assessing their potential as DNA-vaccines to be used *in vivo*. Two synthetic gene variants were constructed, and together with the wild-type S-genes from the five hantaviruses of interest, they were separately fused to the regulatory elements needed for expression and detection of the proteins in pro- and eukaryotic systems. To ensure proper expression of the proteins in eukaryotic cells, the constructs were transfected into COS-1 cells and the protein expression was analyzed. For future analysis of immune serum from mice vaccinated with the synthetic and wild-type genes, it is important to produce and purify the corresponding proteins from bacteria. The synthetic proteins were successfully expressed in eukaryotic cells, indicating their potential as antigens following DNA-vaccination. Intriguingly, when the synthetic proteins were expressed in bacteria, they were extremely stable. The basic idea of genetic vaccination is that genes encoding the antigen is delivered to the cells of the vaccinee were the antigen is produced. Therefore, it follows that not only the antigentic properties of the antigen in question, but also the stability of the expressed protein is important for the generation of a strong and long-lasting immune response. Hence, the unexpected stability of the synthetic proteins (hopefully also observed when the proteins are expressed in the mammalian host) is very encouraging for future use of the synthetic genes in DNA-vaccinations. Expression of the wild-type N-proteins followed the same pattern in bacteria and in eukaryotic cells. The SEOV, SNV and PUUV N-proteins were abundantly expressed by the cells and immunofluorescence analysis of transfected COS-1 cells revealed that the proteins were expressed by most cells. On the contrary, the HTNV and DOBV N-proteins were rapidly degraded in bacteria and also the expression level of HTNV N and DOBV N in eukaryotic cells were low. However, immunofluorescence analysis revealed that the proteins were expressed at an acceptable level, but that only a fraction of the cells expressed the proteins. This could be explained by a reduced transformation-frequency for the HTNV S and DOBVS constructs. Since all attempts to reduce degradation of the HTNV N and DOBV N proteins failed, the best way to deal with the problem might be to generate new clones, although the sequence of all clones are correct and they all result in protein of the expected size.

In the next phase of our study, the synthetic and wild-type genes will be evaluated *in vivo* by DNA-vaccinating (gene-gun) BALB/c mice with the eukaryotic constructs. Blood samples will be withdrawn during the vaccination period and serum will be analyzed for cross-reactivity. Proteins and peptides produced from the synthetic- and the corresponding wt S-genes will be expressed in and purified from *E. coli* cultures and used in the analysis and evaluation of the sera obtained

from the vaccinated mice. Analyses will primarily be carried out by Enzyme-linked immuosorbent assays (ELISA), where the purified proteins will be used as antigen. The relative binding of the antibodies towards the synthetic- and the corresponding N-proteins will be determined, followed by an evaluation of the degree of cross-reactivity between the antibodies obtained. Based on the results, conclusions will be drawn of the potential use of the synthetic genes as a hantavirus vaccine with increased cross-reactivity and protective efficacy.

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Report title

Creation of synthetic genes in order to increase the cross-reactivity and protective efficacy of Hantavirus DNA-vaccines

Abstract (not more than 200 words)

Annually, hantaviruses infect more than 150,000 people worldwide, resulting in thousands of deaths, why prophylactic treatment such as vaccination against human pathogens among the hantaviruses is highly desirable. The immunological cross-reactivity and high genetic conservation between these viruses could be one key towards a common vaccination strategy. In this study, we have initiated an investigation exploring the possibility of creating one “universal” DNA-vaccine against several of the human pathogenic hantaviruses. We have constructed two synthetic genes, multi-epitope constructs, derived from proposed B- and T-cell epitopes of the S-genes from five different hantaviruses. The epitopes have been modified for increased cross-reactivity by individual amino acid substitutions towards common consensus sequences within the proposed epitope. Furthermore, the individual epitopes were flanked with immuno-stimulatory DNA sequences (CpG) for enhanced immunogenicity. This report contains the initial results from the efforts of exploring the possibility of a multi-epitope vaccine with an enhanced cross-reactivity. The synthetic genes and the corresponding full-length S-genes were successfully expressed in bacteria for the production of antigens used to evaluate the immune response obtained in animal model systems. The genes were also expressed in eukaryotic cells, indicating the possibility of *in vivo* expression of the encoded antigens in vaccinated animals. Interestingly, the synthetic proteins turned out to be very stable, which is favorable for the generation of a strong and long-lasting immune response.

Keywords

Hantavirus, cross-reactivity, Puumala virus, Nucleocapsid protein

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Syntetiska gener skapade för att öka korsreaktivitet och skyddande förmåga hos Hantavirus DNA-vaccin

Sammanfattning (högst 200 ord)

Hantavirus infekterar årligen mer än 150 000 människor världen över. Eftersom tusentals människor dör till följd av infektionerna är profylaktisk behandling, såsom vaccinering, mot dessa hantavirus av stort folkhälsointresse. Immunologisk korsreaktivitet samt en stor genetisk likhet mellan dessa virus skulle kunna vara nyckeln till en gemensam vaccinations-strategi. Den här studien syftar till att utreda möjligheten att konstruera ett ”universellt” DNA-vaccin som är verksamt mot flera humanpatogena hantavirus. I denna första del av studien har vi konstruerat två syntetiska gener, multi-epitop konstrukt, utifrån förutspådda B- och T-cells epitoper från S-generna hos fem olika hantavirus. För att efterlikna gemensamma konsensussekvenser och ge en ökad korsreaktivitet har epitoperna modifierats genom aminosyra substitutioner. Vidare har de individuella epitoperna flankerats med immunstimulerande DNA-sekvenser (CpG-motif) för ökad immunogenicitet. Syntetiska gener samt motsvarande full-längds S-gener har med framgång uttryckts i bakterier, för produktion av antigen att användas i analysen av immunsvar i djurmodell. Generna har även uttryckts i eukaryota celler för att utvärdera möjligheterna till *in vivo* expression av generna i vaccinerade djur. Glädjande nog visade sig de syntetiska proteinerna vara mycket stabila, vilket är en klar fördel när det gäller att generera ett starkt och långvarigt immunsvar. Den här rapporten sammanfattar den första delen av studien och innehåller *in vitro* resultat, som ligger till grund för en påföljande *in vivo*-studie.

Nyckelord

Hantavirus, korsreaktivitet, Puumala virus, Nukleokapsidprotein

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