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Immunogenic epitopes of Hantaviruses' N protein are restricted to conserved regions

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1. ABSTRACT

The *Bunyaviridae* virus family is composed by five genera, of which the *Hantavirus* genus is one of the most important representatives. Occasionally, these viruses can be transmitted to humans, giving rise to severe diseases that present high mortality rates. We analyzed the amino acid sequences of the nucleocapsid (N) proteins of 34 different hantaviruses to investigate the potential mechanisms involved in immunogenicity against hantaviruses. Immunogenic epitopes described in the literature through experimental analyses for Sin Nombre (SNV), Puumala (PUUV), and Hantaan (HTNV) viruses' species were retrieved. We identified and characterized the regions believed to be responsible for the induction of immune response in hosts. We found that N protein epitopes described in the literature for PUUV, SNV and HTNV viruses are all located in highly conserved regions of the protein. The high conservation of these regions suggests that a cross-reactive immune response among different hantaviruses can be induced.

2. INTRODUCTION

The *Bunyaviridae* family includes more than 300 viral species distributed as five genera (1), and most of these viruses are transmitted by arthropods. Members of the *Hantavirus* genus, first isolated in 1978, are enveloped viruses with 80-140 nm virions and are the only non-arbovirus group, i.e. not transmitted by arthropods, in the *Bunyaviridae* family (2). Hantaviruses genome consists of three negative strand RNA segments: L (large), M (medium), and S (small) (3). The L segment encodes an RNA-dependent RNA polymerase; the M segment encodes two glycoproteins (Gn and Gc) inserted in the viral envelope membrane; the S segment encodes a nucleocapsid protein (~50 kDa) also referred to as N protein (4). Because the N protein is an internal virion component, it suffers less selective pressure from immune responses than the envelope glycoproteins (5) what could explain the high structural conservation of hantavirus N protein (6). Also, the N protein is highly abundant during the course of hantavirus infection, (7) and cross-reactive immune

Table 1. Immunogenic epitopes for each N protein region

Specie	Sequence1	Region1	Reference
SNV	^{94}SSLRYGNV^{101}	94 – 101	(12)
PUUV	^{94}SSLRYGNV^{101}		
SNV	^{180}SMPTAQSTM^{188}	180 -188	(12)
PUUV	^{180}SMPTAQSTM^{188}		
SNV	^{218}PVMGVIGFS^{226}	218 – 226	(12)
PUUV	^{218}PVMGVIGFS^{226}		
SNV	^{332}AILQDMRNT^{340}	332 – 340	(12)
HTNV	^{221}SVIGFLAL^{228}	221 – 228	(25)
HTNV	^{328}LGAFFSIL^{335}	328 - 335	(25)

Abbreviations: SNV: Sin Nombre virus; HTNV: Hantaan virus; PUUV: Puumala virus.[1] The numbers refer to the first and the last amino acid of the sequence.

responses have already been observed in different hantaviruses species (8-12).

In humans, hantaviruses can cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), which can reach case fatality rates even higher than 50% (13). HFRS is more prevalent in Asia and Europe, while HPS occurs mainly in the Americas (14, 15). Although efforts have been made in the last years (16), no effective and safe vaccines have been developed until now (17).

In order to understand hantavirus immunogenicity we searched the literature for epitopes and N protein sequences from different hantaviruses species. Immunogenic epitopes from three different hantaviruses species – Sin Nombre virus (SNV), Puumala virus (PUUV) and Hantaan virus (HTNV) – all of which described through experimental analyses, such as ELISPOT and chromium-release assay, were retrieved from Immune Epitope Data Bank (IEDB) and analysed. To investigate if these regions are specific to the *Hantavirus* genus, we created a motif of each region and searched a non-redundant protein databank for similar proteins. Also, we analysed the entire N protein through sliding window method and compared to proteins derived from rodents. This would reveal similarities between self (rodent) and non-self (the N protein) proteins and would allow to examine whether the immune system chooses regions in the N protein that are different from the host, consequently minimizing the risks of autoimmune episodes. Finally, taking into account the immunological differences between humans and rodents, we submitted the N protein to the human antigen processing pathway, in order to suggest the use of such epitopes in a human vaccine.

3. MATERIALS AND METHODS

3.1. Search for hantaviruses epitopes described in the literature

A search in the literature for immunogenic epitopes derived from hantaviruses nucleocapsid protein was performed. Only epitopes that have been proved to elicit a predominantly CD8+ T cell and strong murine immune response *in vivo* or *in vitro* assays were selected and included in our analysis. A total of nine epitopes from three different hantaviruses species – SNV, PUUV and HTNV – were retrieved (Table 1). Also, several human hantaviruses epitopes from different species were retrieved from Immune Epitope Data Bank (18) and colocalized in the alignment.

3.2. N protein search and alignment

All selected epitopes (8 to 10 amino acids in size) from murines (Table 1) were submitted to a *BLASTp (*19) in order to identify similar sequences. This approach allowed us to retrieve the N protein from several hantaviruses species. We used the default parameters of the software, except "*Word size*" (value = 2) and "*Max target sequences*" (value = 250), which were used to optimize the search. The results were filtered considering the original organism where the sequence was described. At this point of the analysis only non-redundant and complete N protein sequences were considered as valid results. Finally, 34 completed sequences from different species of the *Hantavirus* genus were retrieved and aligned using the Muscle software (20). The visualization and identification of conserved regions were performed using the GeneDoc software *(*http://www.psc.edu/biomed/genedoc). The 34 different species and their respective NCBI accession numbers are: Sin nombre (Accession No.: AAG03030.1), Puumala (Accession No.: CAB65379.1), Hantaan (Accession No.: AAW02944.1), Seewis (Accession No.: ABR24795.1), Cao Bang (Accession No.: ABR29825.1), Soochong (Accession No.: AAT78474.1), Amur (Accession No.: BAD04845.1), Saarema (Accession No.: CAE83602.1), Seoul (Accession No.: AAK96243.1), Thailand (Accession No.: CAL37107.1), Muju (Accession No.: AAZ67072.1), Topografov (Accession No.: CAB42097.1), Vladivostok (Accession No.: BAA25145.1), Tula (Accession No.: CAA11466.1), Malacky (Accession No.: CAA92340.1), Neembucu (Accession No.: ABC70873.1), Bermejo (Accession No.: AAL82648.1), Lechiguanas (Accession No.: AAL82649.1), Andes (Accession No.: AAK11225.1), Oran (Accession No.: AAL82650.1), Araucaria (Accession No.: AAW57482.1), Pergamino (Accession No.: AAL82652.1), Maciel (Accession No.: AAL82651.1), Alto Paraquay (Accession No.: ABC70872.1), Rio Mamore (Accession No.: AAC58450.1), Laguna Negra (Accession No.: AAB87601.1), Maporal (Accession No.: AAP92140.1), Choclo (Accession No.: ABB90557.1), Catacamas (Accession No.: ABB83548.1), Bayou (Accession No.: AAA61691.1), Muleshoe (Accession No.: AAD00082.1), Cano Delgadito (Accession No.: AAB71815.1), Limestone Canyon (Accession No.: AAK49899.1) and Thottapalayam (Accession No.: AAS19458.1).

3.3. Sliding window method

As already described (21), we used the sliding window method to compare the N protein from hantaviruses with self rodent proteins. The N protein sequence of SNV was used as reference. A sliding 10-residue window was set to generate a BLAST score against host sequences (*Rodentia* databank) to each N protein decamer. We assumed arbitrary values of up to 70%, 71-80%, and 81-100% for low, intermediate, and high similarity, respectively.

3.4. Pattern search

To further analyze the corresponding regions of the described epitopes – four regions - we generated

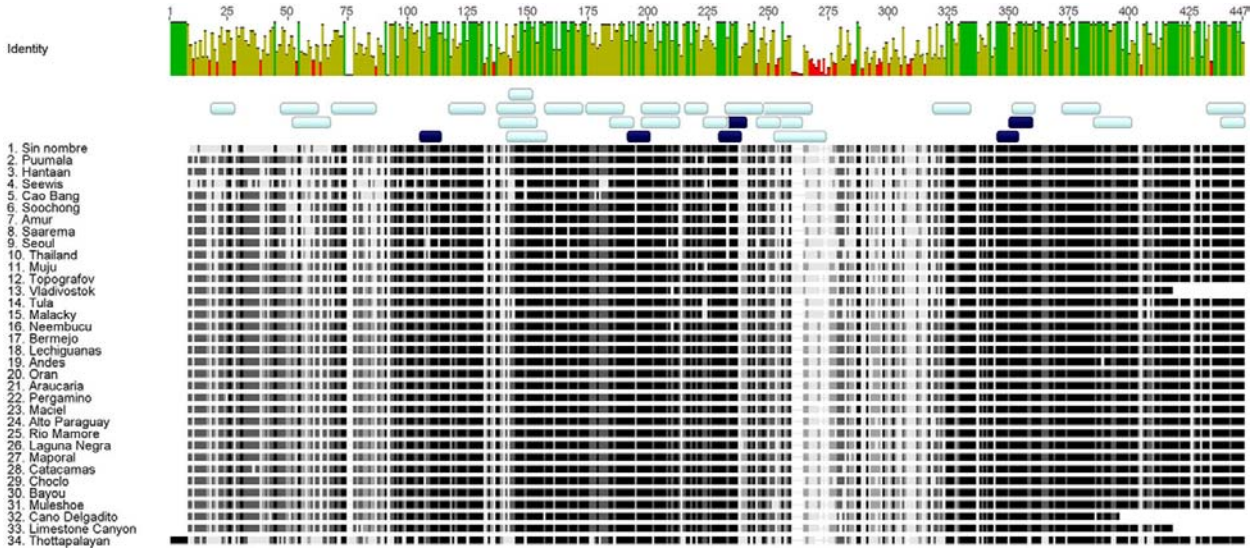

Figure 1. Multiple alignment of sequences, evidencing the conserved regions pattern. Alignment of N protein amino acid sequences from 34 different hantaviruses species. Each box, above the alignment, represents the screened hantaviruses epitopes in murines (dark blue boxes) or in humans (soft blue boxes). On the top, the identity is presented as a histogram. Values below 50%, ranging from 51% to 99% and 100% of identity are depicted by red, mustard and green bars, respectively.

consensus motifs, based on the alignment of the N protein of the 34 hantaviruses members (Table 1). A search was performed with each motif as the entry sequence. In this search, all non-redundant proteins available in the Swiss-prot data bank were investigated through the PatternSearch tool from the Max Planck Institute (http://toolkit.tuebingen.mpg.de/pat-search).

3.5. Epitope generation in humans

To investigate if the N protein epitopes described in the literature could be generated during an immune response in humans we performed an *in silico* approach simulating the steps of the antigenic processing pathway. We used tools for each specific steps to this pathway: proteasome cleavage - Netchop 3.0 (22); TAP transporting – TapPred (23) and MHC binding - NetMHC 3.2 (24). Based on the prevalence of the MHC alleles in different human populations we chose the following HLAs in NetMHC analysis: HLA-A*0201, HLA-A*0301, HLA-A*1101, and HLA-B*07.

3.6. Human autoimmune propensity investigation

We performed a comparison between N protein epitopes and human proteins in order to investigate if these sequences could elicit a cross immune response against related self-proteins in humans. Therefore, a *BLASTp* against *Homo sapiens* protein data bank (NCBI) was performed. The *BLASTp* parameters were maintained as described above. Human proteins presenting similarity with the N protein epitopes higher than 75% were downloaded, and underwent the same approach as described in section 3.5.

4. RESULTS AND DISCUSSION

In the present work we analysed the amino acid sequence of the N protein from different hantaviruses species in order to investigate the potential mechanisms involved in hantaviruses immunogenicity. Immunogenic hantaviruses epitopes, described in the literature, were used as the starting point of our work, since they were defined by *in vitro* or/and *in vivo* experimental approaches, and therefore represent the immunogenic regions of the N protein that are more relevant in the induction of the immune response against hantaviruses in murines. In this context, our approach based on bioinformatics tools is greatly strengthened by the usage of experimental data.

Immunogenic peptides already described in murines were identified from three hantaviruses species: PUUV, SNV, and HTNV (Table 1) (12, 25). When these epitopes were submitted to a BLASTp search against the whole NCBI protein databank, we observed that 210 out of the first 250 output sequences presented high similarity degree with N protein sequences from *Hantavirus* genus members. This indicates that these sequences are characteristic of this viral group. This analysis allowed the identification of N protein sequences from 34 different members of the *Hantavirus* genus.

Considering that tables of alignments are able to highlight amino acid conservation patterns with high confidence, the 34 N protein amino acid sequences were aligned to provide an overview of N protein from various species of the Hantavirus genus. We found that murine epitopes are concentrated in more conserved regions (Figure 1). Contrastingly, human epitopes presented a more disperse localization along the hantavirus N protein. This is the first work to correlate hantaviruses conserved regions with immunogenic epitopes.

Many efforts have been undertaken by research groups. However, there is still no safe and effective vaccine

Table 2. Output sequences containing the consensus motif for each N protein region

Motif	Region	Output sequences containing the motif
		All sequences on GenBank / only hantaviruses sequences
S (SMAT) (LP) (RS) Y G N (VTI)	94 - 101	391/389 (99.5%)
S (ML) P (TN) (AS) Q S (TS) (MI)	180 - 188	490/489 (99.8%)
P V M (GS) V (IV) G F (SLPMAGNQ)	218 - 226	557/553 (99.3%)
(AS) (IVL) (LM) Q D (MI) R (NG) (TG)	332 - 340	337/335 (99.4%)
(SG) V (IV) G F (SLPMAGNQ) (FAVSH) (FLI)	221 - 228	917/543 (59.2%)
L G A F (FL) (SA) (IVL) (LM)	328 - 335	435/369 (84.8%)

licensed by the World Health Organization, neither a specific antiviral treatment. Some approaches are being used more than others. Among these approaches, it should be highlighted the use of chimeric viruses and virus-like particles, recombinant proteins, genetic vaccines and inactivated virus, which are already being used in humans in some Asia countries, such as China and North Korea (26, 27).

The advances in hantavirus vaccinology is evidencing that both, humoral and cellular immune response, either against glycoproteins Gn/Gc or N protein as well, are very important for the viral clearance. In fact, it is believed that Gn/Gc elicits mainly the humoral response (28, 29), while the N protein elicits mainly the cellular immune response (30, 31). The cellular immune response has proved importance for the hantavirus clearance in humans, however the "Janus faced" of this response can be a problem, since a dual effect can eliminate the pathogen or, controversially, exacerbate the pathogenesis (32, 33). In this sense, we could infer that the high dispersion of immunogenic epitopes in humans, contrasting with the fact that murine epitopes are limited to conserved regions of the N protein, can be one of the factors responsible by the increase of the cellular immune response, enhancing the inflammatory scenario.

In this way, the identification of possible epitopes recognized by humans into the conserved regions of the N protein could bypass the problems of such an inefficient response and would potentially be an interesting strategy in vaccine development.

An immune response directed against a region that presents low variability among different viruses can potentially allow the occurrence of cross-reactivity. In fact, Maeda *et al* (2004) already showed the existence of cross-reactivity directed against different regions of the N protein (N_{94-101}, $N_{180-188}$ and $N_{218-226}$) between the SNV and PUUV viruses (12). We believe that alterations in these regions affect negatively the viral infectivity or maintenance in the host and, therefore, although under a selective pressure as targets to immune responses, these regions do not show high variability among the different viruses species. In fact, it is known that the N_{1-125} region, which encompasses the $PUUV_{94-101}$ and SNV_{94-101} epitopes, is involved in protein oligomerization and that the region encompassing the $PUUV_{180-188}$ and $SNV_{180-188}$ epitopes is a RNA binding region (34).

The sliding window analysis evidenced that decamers superposing the epitope regions presented low similarity levels (up to 70%) with protein sequences from rodents, pointing towards a long-term coevolution between rodents immune system and hantaviruses. The SNV_{94-103} decamer, which contains the epitope SNV_{94-101}, presented less than 60% of similarity with any of the sequences from Rodentia. All the decamers that superposes the region of the epitope SNV_{94-101} (SNV_{85-94}; SNV_{86-95}; …; $SNV_{101-110}$) also showed low similarities with rodent proteins, reaching a maximum value of 70%. Similar results were obtained to the $SNV_{180-189}$, $SNV_{218-227}$, and $SNV_{328-335}$ regions.

To investigate if the immunogenic epitopes from N protein described in table 1 are specific to hantaviruses species we created a consensus motif of each region based on the alignment of the N protein of the 34 hantaviruses members. Each consensus motif was used as an input sequence against all sequences in the GenBank through the PatternSearch tool (35). These results are summarized in Table 2. The outputs from the PatternSearch tool suggest that the consensus motifs are characteristic (or even exclusive) of the Hantavirus group. The only exception was the consensus motif for the region 221-228. It is important to point out that this region is less conserved than the other N protein regions, as can be observed both in Figure 1 and Table 1, and contains proteins from different sources, ranging from organisms like bacteria and fungus to proteins from *Homo sapiens* (less than 1%).

Considering the development of a vaccine to be used in humans, we assessed the possibility that the N protein epitopes described in the literature could be generated during an immune response in humans through the intracellular antigen processing pathway. We submitted the original protein sequences from SNV, PUUV and HTNV to immunoinformatic tools (36, 37). First, we analysed the complete N protein sequences with Netchop 3.0 (22), which simulates the immunoproteasome cleavage. Subsequently we submitted the resulting epitopes to TapPred (23), in order to evaluate the peptide binding to the transporter associated with antigen processing (TAP). Finally, we evaluated the epitope binding affinity for the high prevalent human alleles HLA-A*0201, HLA-A*0301, HLA-A*1101, and HLA-B*07, using the NetMHC 3.2 (24).

The analysis of the *in silico* proteasomal cleavage (Netchop) and TAP affinity (TapPred) showed that, although the correct requirements for proteasomal cleavage were present in some murine epitopes of the N protein, only the epitope SVIGFLALA ($N_{221-229}$) had all the predicted requirements to be generated and presented for a T lymphocyte in humans. All results are presented in Table 3. This suggests that immunogenic epitopes for the murine immune system will not necessarily be generated in

Table 3. TapPred and NetChop predictions for TAP binding and C-terminal precise cutting, respectively

Species	Sequence	NetChop (C-terminal Precise Cutting)	TapPred (Predicted Affinity)	NetMHC affinity (nM)[1]			
				HLA-A*0201	HLA-A*0301	HLA-A*1101	HLA-B*0702
SNV	^{91}KSSLRYGNV^{99}	0.114010	High	15089	17381	19792	22930
SNV	^{92}SSLRYGNVL^{100}	0.925992	Intermidate	18668	22460	21336	10917
SNV	^{178}SMPTAQSTM^{186}	0.977767	Intermidate	4221	20782	24776	18394
SNV	^{216}PVMGVIGFS^{224}	0.036510	Low or undetectable	20904	19477	24749	24774
SNV	^{330}AILQDMRNT^{338}	0.024778	High	17525	22468	29295	25590
PUUV	^{93}RSSLRYGNV^{101}	0.111766	High	17364	17202	19128	20101
PUUV	^{94}SSLRYGNVL^{102}	0.869308	Intermidate	18668	22460	21336	10917
PUUV	^{180}SMPTAQSTM^{188}	0.976805	Intermidate	4221	20782	24776	18394
PUUV	^{218}PVMGVIGFS^{226}	0.034059	Low or undetectable	20904	19477	24749	24774
HTNV	^{220}MSVIGFLAL^{228}	0.964342	Intermidate	8573	18622	17295	4695
HTNV	^{221}SVIGFLALA^{229}	0.707558	High	113 (Weak Binding)	16908	4641	19615
HTNV	^{327}ELGAFFSIL^{335}	0.870844	Low or undetectable	6608	20665	25331	22379
HTNV	^{328}LGAFFSILQ^{336}	0.033648	Intermidate	22791	19690	22075	27706
Homo sapeins (RNT1)	^{548}SVVAYLALA^{556}	0.396658	High	123 (Weak Binding)	16343	9098	23252

[1] Affinity as IC50 value in nM. The value is inversely proportional to the binding affinity.

humans. Such differences can be explained by the different interactions of the murine and the human immune systems to hantaviruses. Considering that rodents are the natural hosts for hantaviruses, we can envisage the superposition of two scenarios, where the murine immune system efficiently selects good targets to minimize the morbidity associated to the infection and simultaneously selects strains that present low lethality rates, therefore favoring hantaviruses maintenance (38).

After the selection of immunogenic epitopes (SVIGFLALA - $N_{221-229}$) that can be generated and presented by the human immune system, we performed a second step – the search for similar human sequences that can trigger cross-reactive events and the propensity to autoimmune disorders. When we compared the hantavirus immunogenic epitope (SVIGFLALA - $N_{221-229}$) with all human proteins available at GenBank databank, only one protein (RTN1) presented a region (SVVAYLALA - $RTN1_{548-556}$) with similarity to the foreign epitope higher than 80%. The RTN1 protein was submitted to imunoinformatics tools, which simulate the complete antigen processing pathway, in order to evaluate if the human sequence SVVAYLALA 548-556 will be generated and presented by the whole process. Although the sequence SVVAYLALA has a high affinity by TAP, the protein RTN1 has no predict cleavage site flanking the region, which render more difficult the generation of this epitope. Besides, the sequence only can be presented by the HLA-A*0201 allele, as a weak ligand.

In conclusion, we postulate that the murine immune system is preferentially directed against hantaviruses epitopes from conserved regions and that do not present a significant identity to self proteins. This phenomenon minimizes the risk of autoimmune responses. This idea is supported by our results from the comparison between the N protein and proteins from the *Rodentia* family (the natural hosts of hantaviruses) obtained through the sliding window method. Also, although hantaviruses use mechanisms to evade immune responses (as any other pathogen), the potential to change some protein regions is not unlimited, since this alteration can negatively affect the viral infectivity or maintenance in the host. Therefore, although under a selective pressure as targets to immune responses, some proteins do not show high variability across different viruses and can therefore be considered potential candidates in vaccine development strategies. The development of reliable and effective vaccines is quite important, and to achieve such objective, we should understand the mechanisms underlying the selection of viral targets by the immune system. The high conservation of the immunogenic regions of hantaviruses, evidenced in our study, suggests that a cross-reactive immune response among different hantaviruses can be induced (39).

Also, the lack of similarity among the tested epitopes and humans points to these as potential targets for vaccine development, with reduced risk of adverse effects due to autoimmune responses.

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Abbreviations: BLAST: Basic Local Alignment Search Tool, ELISPOT: Enzyme-linked Immunosorbent Spot, HCPA: Hantavirus Pulmonary Syndrome, HLA-A: Human Leukocyte Antigen, HFRS: Hemorrhagic Fever with Renal Syndrome, HTNV: Hantaan virus, IEDB: Immune Epitope Database, L: Large, M: Medium, MHC: Major Histocompatibility Complex, N: Nucleocapsid, PUUV: Puumala virus, S: Small, SNV: Sin Nombre virus, RNA: Ribonucleic Acid, TAP: Transporter Associated with Antigen Processing.

Key Words: *Hantavirus* genus, Immunogenicity, *Bunyaviridae* family, Nucleocapsid protein, Immunoinformatics

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