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Virology 334 (2005) 319–326
VIROLOGY
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Human and rodent humoral immune responses to Andes virus structural proteins

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Received 19 October 2004; returned to author for revision 16 November 2004; accepted 26 January 2005

Abstract

In the present work we identified B-cell epitopes recognized by sera of humans and rodents naturally infected with Andes virus, a hantavirus present in Chile and Argentina. Analysis of patient and rodent sera with overlapping peptides revealed 21 human and rodent epitopes on the three structural proteins. Whereas in the nucleoprotein the region comprising aa 248–260 was shown to be the key determinant of human sera, the major antigenic site of rodent antibody reactivity is located at aa 326–338. In G1, the main epitope recognized by human sera was mapped to aa 14–26, while rodent antibodies bound predominantly to aa 599–611. In contrast, humans and mice had strong responses to three regions in G2 (aa 691–703, aa 918–930, aa 955–967), of which the last two are associated with neutralization of Hantaan virus. This insight affords important information for the development of immunotherapies for the acute phase of hantavirus cardiopulmonary syndrome.
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Keywords: Hantavirus; Linear B-cell epitopes; Neutralizing antibodies

Introduction

Hantaviruses form enveloped, spherical virions of 80–140 nm in size. Like all members of the Bunyaviridae family they possess three structural proteins: the glycoproteins 1 and 2 (G1 and G2), which are inserted into the viral envelope membrane, and the nucleocapsid protein (N), associated to the viral genomic ssRNA(−). In humans they cause two severe and often fatal diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). The transmission to man of these rodent borne diseases has been associated with inhalation of aerosols from excreta of infected mice (Nuzum et al., 1988). In Argentina and Chile approximately 1000 cases of HCPS, reaching mortality rates up to 40%, have been detected since 1993, when the new hantavirus variant named Andes (ANDV) was first identified as the responsible etiological agent (López et al., 1996).

The ANDV has called attention by several particular features. It is the only one known for which evidence of inter-human transmission has been reported (Padula et al., 1998). In addition, together with one other South American hantavirus (Maporal virus) it is known to produce a fatal disease in Syrian hamsters (Hooper et al., 2001; Milazzo et al., 2002). Lastly, it has been detected in various rodent species, supporting the idea that one virus species may infect more than one rodent species (Jay et al., 1997; Kuenzi et al., 1999; Morzunov et al., 1998; Plyusnin and Morzunov, 2001). The principal reservoir of ANDV is *Oligoryzomys longicaudatus* (Levis et al., 1998); however, it also has been detected with lower incidence in *Abrothrix longipilis*, *Abrothrix olivaceus*, *Phyllotis darwini*, and *Loxodontomys*

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0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.01.031

micropus in Chile (Sportorno et al., 2000) and in *Oligoryzomys chacoensis* and *Akodon varius* in Argentina (Calderon et al., 1999). The mechanism by which hantaviruses persist in their natural reservoirs has been suggested to arise by escape of specific $CD8^+$ immune surveillance (Araki et al., 2004). In contrast, humoral immune responses appear to be important in conferring protection against viral infection (Arikawa et al., 1992; Zhang et al., 1989). In persistently infected mice, the observed strong neutralizing antibody responses (Botten et al., 2003; Kariwa et al., 1996; Lee et al., 1981) may be essential to suppress hantavirus dissemination and consequently to avoid fatal illness (Araki et al., 2004). In accordance with the former, HCPS patients with high neutralizing antibody titers in the acute phase of infection undergo a more favorable clinical outcome than patients with lower titers (Bharadwaj et al., 2000, Galeno et al., unpublished data). Hantavirus neutralizing activity has been related to antibodies directed to the surface proteins, since monoclonal antibodies to G1 and G2, but not to N, have been shown to neutralize viral infection in vitro (Dantas et al., 1986). Hantavirus infections induce antibodies predominantly to N, followed by the G proteins (Kallio-Kokko et al., 2001; Sjölander et al., 1993). B-cell epitopes involved in neutralization of Hantaan (HTNV) and Puumala virus (PUUV) have been studied using neutralizing monoclonal antibodies (Hörling and Lundkvist, 1997; Koch et al., 2003; Liang et al., 2003; Wang et al., 1993), while still no data is available about the clearance of HCPS causing viruses.

For the development of immunotherapies for HCPS patients, it is important to understand the humoral immune responses of infected humans and infected rodents. For this aim here we investigate differences of humoral immune responses between ANDV-infected humans and rodents and between patients with different clinical outcomes. We performed a fine mapping of B-cell epitopes on the three structural proteins of ANDV recognized by sera of infected individuals and compared them with their clinical evolution and neutralizing antibody titers. Our results show the existence of two immunodominant regions in G2 common among human and rodent immune responses, while the major antigenic domains in N and G1 differ between man and mice. In contrast, no significant epitopic variations could be distinguished related to the different clinical outcomes of patients or neutralizing antibody titers. Nevertheless, among the 13 predominant epitopes identified on the glycoproteins, five are homologous to regions in HTNV or PUUV, which have been associated by others with viral neutralization.

Results and discussion

Characterization of immune sera

Sera of 23 HCPS patients with different clinical outcomes were collected between days 2 and 210 after the

Table 1
Characterization of HCPS patient sera samples

Sera number		Patient		Days of evolution[a]	Outcome of HCPS	Egress	Titer of IgM anti N	Titer of IgG anti N	Neutralizing Ab titer[b]
		Gender	Age						
1	2000-7367	M	30	4	Severe	Deceased	6400	400	50
2	2000-640	M	48	4	Severe	Deceased	6400	0	100
3	2001-4741	M	47	5	Severe	Survived	1600	800	100
4	2001-5457	F	55	4	Severe	Survived	6400	0	200
5	2001-1133	F	26	6	Severe	Survived	6400	0	200
6	2001-4890	M	34	8	Moderate	Survived	6400	0	200
7	2001-2725	M	23	6	Moderate	Survived	6400	400	200
8	2001-2235	F	42	7	Mild	Survived	6400	1600	200
9	1997-703327	n.i.	n.i.	>90	n.i.	Survived	400	400	200
10	2001-4723	M	48	5	Mild	Survived	6400	1600	300
11	2001-3970	M	40	5	Severe	Survived	6400	0	400
12	2001-1977	M	24	7	Severe	Survived	1600	0	400
13	2000-6895	M	n.i.	5	Severe	Survived	6400	6400	400
14	2001-645	M	32	10	Moderate	Survived	6400	400	400
15	2000-3500	M	49	7	Moderate	Survived	6400	1600	400
16	2001-11629	M	26	9	Moderate	Survived	6400	6400	800
17	2001-1981	M	17	6	Mild	Survived	6400	0	800
18	2001-2840	M	26	6	Moderate	Survived	6400	0	1600
19	2001-9423	F	19	210	n.i.	Survived	0	6400	1600
20	1997-703328	n.i.	n.i.	>90	n.i.	Survived	1600	1600	1600
21	1997-703329	n.i.	n.i.	>90	n.i.	Survived	0	6400	2500
22	2000-9844	M	34	2	Moderate	Survived	6400	0	3200
23	2000-11457	F	33	21	Mild	Survived	6400	6400	3200

Titers are reciprocal of dilutions. N = Hantavirus nucleoprotein; M = Male; F = Female; n.i. = no information.
[a] Day in which sample was taken counted from the beginning of symptoms.
[b] 80% focus reduction.

onset of symptoms. As seen in Table 1, the patients' ages ranged between 17 and 55 years (average of 34 years). The majority of the patients were of male gender (15 of 23 patients). Table 1 also shows the clinical characteristics of the patients, including serological information such as the titer of N-specific IgG and IgM examined by ELISA and the neutralizing antibody titer determined by focus reduction neutralization assays. Of these sera, 21 contained N-specific IgM, while 14 contained N-specific IgG, indicating that the majority were derived from patients in the acute phase of disease, while only two sera were from patients in the convalescence phase. Furthermore, 21 samples derived from surviving patients, while two were from deceased patients. Notably, the deceased patients and most of the severe patients show very low neutralizing antibody titers, suggesting, as reported previously (Bharadwaj et al., 2000, Galeno et al., unpublished data), their importance in the outcome of HCPS. However, due to the small number of patients examined and the lack of information of the clinical outcome in five cases, no clear dependence could be deduced. Rodent blood samples were also analyzed. The samples were derived from ANDV-infected animals captured in Chile, six were from *O. longicaudatus* and one from *A. longipilis*. All rodent samples studied contained N-specific IgG (data not shown).

Human and rodent nucleocapsid protein B-cell epitopes

HCPS patient sera (n = 23) and sera from naturally infected rodents (n = 7) were analyzed for reactivities against 36 13-mer peptides of the nucleocapsid protein of ANDV CHI-7913 (Tischler et al., 2003). As can be seen in Fig. 1, epitopes recognized by the majority of human immune sera could be located within seven regions distributed over the entire N (aa 1–78, aa 144–169, aa 209–221, aa 248–260, aa 326–338, aa 365–377, aa 404–428). Control sera as well as the detection antibody alone showed no binding (data not shown). Of these seven immunogenic regions, the epitope aa 248–260 located in the middle region of the protein, could be identified as a major antigenic domain since all of the 23 patient sera reacted strongly with this peptide (average score = 1.8). In contrast, rodent immune sera did not react with this region. Instead, the region of aa 326–338 was found to be the major rodent epitope on N (average score = 1.9) (Fig. 1). The remaining reactivity patterns described for humans were also shared by rodents, except the C terminal region aa 404–428. In addition, a slight reactivity of rodent sera could be detected at aa 183–195. Further immunoreactive regions may exist within N, since the peptides were designed in a non-overlapping manner.

Human and rodent glycoprotein B-cell epitopes

To identify B-cell epitopes of ANDV glycoproteins 1 and 2, we first examined the reactivity of four patient sera with 87 non-overlapping 13-mer peptides, which represent the entire ANDV CHI-7913 glycoprotein precursor (GPC) sequence (Tischler et al., 2003). Subsequently a finer mapping of the recognized areas was performed with 43 additional 13-mer peptides overlapping 6–7 amino acids. No reactivity was detected from sera of uninfected humans and rodents (data not shown).

Fig. 1. Identification of ANDV nucleocapsid protein B-cell epitopes recognized by human and rodent sera. Immune sera were incubated with 36 13-mer peptides covering the entire nucleocapsid protein (first column aa 1–428) and reactivity was detected with a chemiluminescence substrate. White squares indicate no reactivity (grade 0), gray squares moderate reactivity (grade 1), and black squares strong reactivity (grade 2) of human (column no. 1–23) and rodent (column no. 24–30) immune sera. Human sera (No. 1–23) are displayed from the lowest to the highest neutralizing antibody titers, as seen with more detail in Table 1.

Fig. 2A shows the results obtained with the 75 13-mer peptides spanning the entire G1 (GPC aa 1–650). Six immunoreactive human epitopes were identified with the 23 patient sera (aa 14–32, aa 46–58, aa 66–104, aa 189–201, aa 215–234, aa 300–344). Only three of these were also recognized by sera of infected rodents (aa 46–58, aa 215–234, aa 300–344), while rodent immune sera also reacted with three other regions (aa 404–416, aa 521–533, aa 599–611). As expected, all epitopes recognized by human sera are located in the predicted ectodomain of this membrane protein. In contrast, two of the three exclusive rodent epitopes (aa 521–546, aa 599–611) are positioned in the predicted cytoplasmatic tail of G1.

Of the epitopes identified in G1, the region of aa 14–26 presented the strongest reactivity with the majority of human sera (average score = 1.5). Given that the glyco-

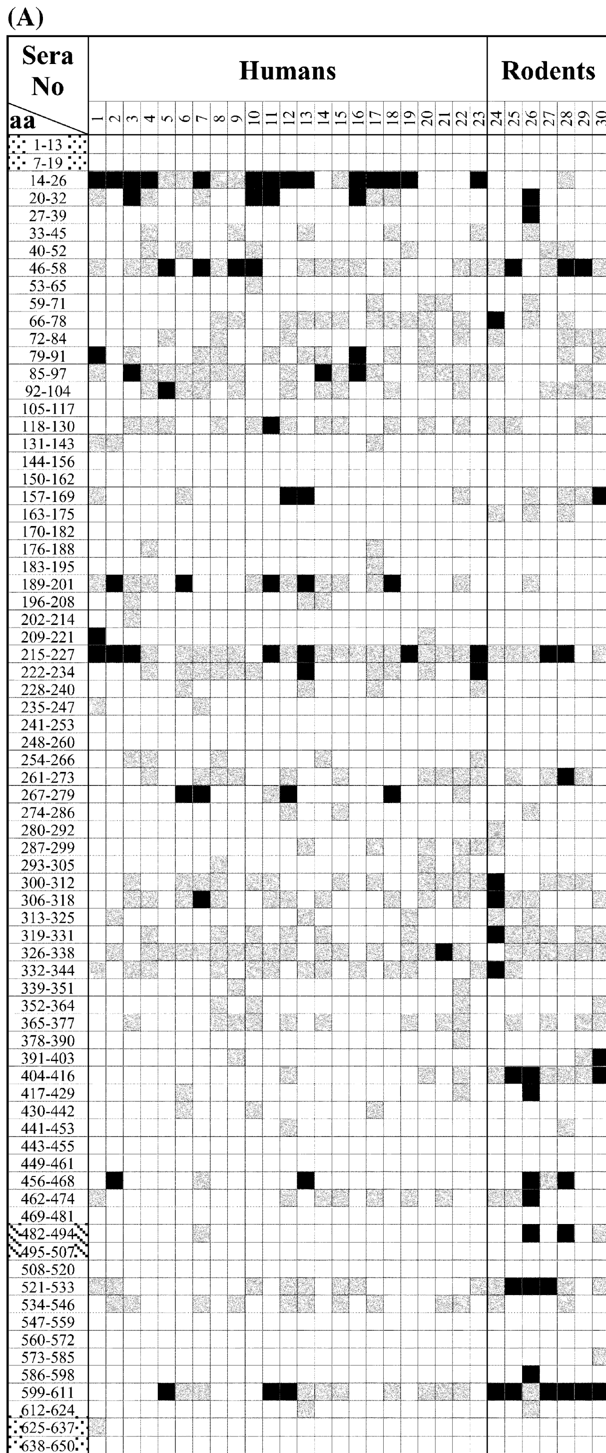

Fig. 2. Identification of ANDV glycoprotein B-cell epitopes of human and rodent sera. Immune sera were incubated with 130 overlapping 13-mer peptides comprising the entire G1 (A) and G2 (B) proteins (first columns aa 1–1138) and reactivity was detected with a chemiluminescence substrate. White squares indicate no reactivity (grade 0), gray squares indicate moderate reactivity (grade 1), and black squares strong reactivity (grade 2) of human (column no. 1–23) and rodent (column no. 24–30) immune sera. Human sera (No. 1–23) are displayed from the lowest to the highest neutralizing antibody titers, as seen with more detail in Table 1. Squares with horizontal lines indicate predicted transmembrane regions, and squares with dots indicate predicted signal peptides.

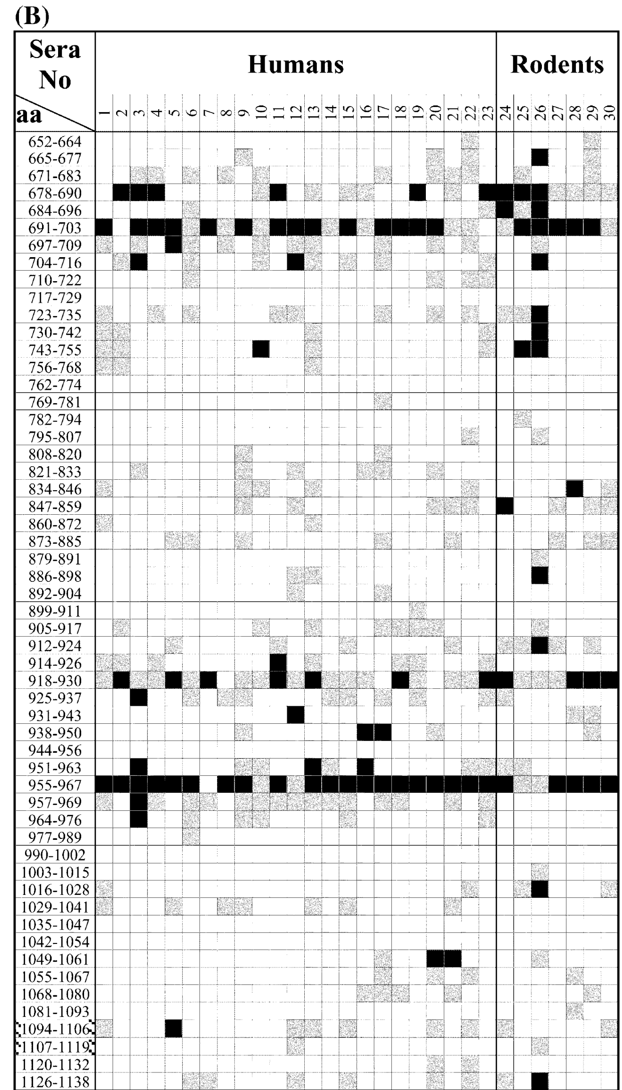

Fig. 2 (*continued*).

proteins of hantaviruses seem to be type 1 oriented membrane proteins (Löber et al., 2001), a signal peptide of about 20 aa length at their amino domain is probably removed. This is supported by a sequencing study, in which the amino terminus of mature G1 of HTNV virus was shown to start at position 18 (Schmaljohn et al., 1987). Therefore, the strong reactivity found in the region of aa 14–26 could represent a linear epitope located at the amino terminus of mature G1, freely accessible to B-cell receptor and antibody binding. In contrast, there was no reactivity of rodent sera to this area. A possible explanation might be that in rodents the G1 protein is processed by different proteases or may be the result of a different immune-repertoire between humans and these particular strains of mice.

The results of our epitope studies of ANDV G1 are consistent with HTNV epitopes recognized by mouse monoclonal antibodies (Wang et al., 1993). As seen in Fig. 3, three identified ANDV G1 epitopes correlate with epitopes of neutralizing monoclonal antibodies against

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ANDV_CHI-7913     212   ISCFFTPKESEQLKVIKTFEGILTKT 237
HTNV_76-118       208   IVCFFVAVKGNTYKIFEQVKKSFEST 233
                                2D5/16D2

ANDV_CHI-7913     297   IVGPITAKVPSTSSTDTLKGTAFAGVPMYSSLSTLVKNADPEFVFSPGIIP 347
HTNV_76-118       294   IVGPANAKVPHSASSDTLSLIAYSGIPSYSSLSILTSSTEAKHVFSPGLFP 344
                                16E6/3D5

ANDV_CHI-7913     401   FNISSPTCLVNKVQRFRGSEQ 421
HTNV_76-118       398   FNITSPMCLVSKQNRFRLTEQ 418
                                         16D2
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Fig. 3. Comparison of G1 epitopes of ANDV and HTNV. Epitopes recognized by ANDV immune sera are underlined and regions recognized by monoclonal antibodies against the HTNV G1 protein are marked by bold underlined letters and the corresponding antibody denomination is indicated below.

HTNV G1 (2D5, 3D5, 16D2, 16E6). Furthermore, the epitopes of non-neutralizing antibodies against HTNV G1 (10F11, 6D4, 8B6) have all been localized in the amino terminal region (aa 163–220) of G1. In this area, we identified a human epitope (189–201) with different grades of reactivity (grades 0 to 2), shown to be recognized in a lesser degree by sera of patients with high neutralizing antibody titers (>1:1600). Although only some of the ANDV G1 epitopes comprise conserved regions within hantaviruses, the strong correlation between epitopes of HTNV and ANDV G1 proteins might reflect a similar accessibility of these regions due to a similar conformation of these envelope proteins. These results suggest that the immunoreactive sites on G1 involved in HTNV neutralization might also be relevant in the neutralization of ANDV.

Fig. 2B shows the results obtained with the 55 13-mer peptides spanning the entire G2 (GPC aa 652–1138). Three major immunogenic regions could be defined by human and rodent immune sera in this protein: aa 678–703, aa 918–930, and aa 955–967. Of these epitopes, the first is located close to the amino terminal of mature G2. Within this region, sera of infected individuals recognized strongly the peptide aa 691–703 (average score of humans is 1.5 and of mice 1.7) and to a lesser degree the peptide aa 678–690 (average score of humans is 0.7 and of mice 1.4), but the majority did not react with the overlapping peptide aa 684–696, suggesting the involvement of two independent epitopes in this region.

In comparison, the very strong recognition of the region aa 955–967 by human and mice sera (20 of 23 human sera with grade 2; average score = 1.8 and 5 of 7 mice sera with grade 2; average score = 1.7) emphasizes the immunodominant role of this epitope within G2. This region shows 91% similarity (76% identity) with the homologous region of HTNV and is reported to be one of two core domains of recognition of a recombinant human neutralizing antibody against the G2 protein of HTNV (Koch et al., 2003) (see Fig. 4). Next to this epitope, the region aa 918–930 also shows a strong B-cell response with human (average score = 1.2) and mice sera (average score = 1.6). This epitope is homologous to a region in HTNV described to be the other core domain recognized by a human neutralizing antibody against HTNV G2 (Koch et al., 2003) (see Fig. 4). Unlike the described HTNV G2 epitope KVMATIDSF (aa 916–924), the sequence recognized by sera of ANDV-infected individuals seems to involve additional amino acids at its C-terminal region for antibody binding, as sera reacted strongly with the peptide RMMATKDSFQSFN (aa 918–930), but in most cases not with the sequence SGYKRMMATKDSF (aa 914–926) (Fig. 2B). The similarity in this region between the ANDV and HTNV sequences amounts to 91% (Fig. 4) compared with 77% of the overall similarity between both G2 proteins. Additional data support the idea that these two highly antigenic regions might be immunodominant also in other hantavirus variants. These sites are located in close vicinity to epitopes of PUUV-neutralizing monoclonal antibodies 1C9 and 4G2 (aa 900–908; aa 913–916, and aa 937 in the ANDV sequence) and of PUUV patient sera (aa 939–959 in the ANDV sequence), identified by neutralizing antibody escape mutants of PUUV (Hörling and Lundkvist, 1997) and phage displayed peptides (Heiskanen et al., 1999), respectively (Fig. 4).

Given that the recombinant human neutralizing antibodies against HTNV G2 also neutralize Seoul and Dobrava virus (Koch et al., 2003), and since the epitopes of neutralizing antibodies directed against PUUV G2 are located in close vicinity, it is quite probable that the two C-terminal major antigenic regions in ANDV G2 identified here might also be involved in the neutralization of ANDV.

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ANDV_CHI-7913  891  TGSFRKICGFATTPVCEYQGNTISGYKRMMATKDSFQSFNLTE  933
PUUV_Sotkamo   898  NGSFRKKCAFATTPVCQFDGNTISGYKRMVATKDSFQSFNVTE  940
HTNV_76-118    893  PGSFRKKCNFATTPICEYDGNMVSGYKKVMATIDSFQSFNTST  931
                              1C9/4G2       1C9  neutr.Fab

ANDV_CHI-7913  934  PHITANKLEWIDPDGNTRDHVNLVLNRDVSFQDLSDNPCKVD  976
PUUV_Sotkamo   941  PHISTSALEWIDLDSSLRDHINVIVSRDLSFQDLSETPCQVD  982
HTNV_76-118    932  MHFTDERIEWKDPDGMLRDHINILVTKDIDFDNLGENPCKIG  979
                      1C9     S1b/S3b          neutr.Fab
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Fig. 4. Comparison of G2 epitopes of ANDV, PUUV, and HTNV. Epitopes recognized by ANDV immune sera are underlined, epitopes recognized by neutralizing monoclonal antibody 1C9 and 4G2 against PUUV G2 protein and PUUV immune sera S1b and S3b are indicated by cursive underlined letters, and human recombinant neutralizing Fab fragments anti-HTNV G2 protein are marked by bold underlined letters.

In summary, our results obtained for the glycoproteins show that sera from ANDV-infected humans recognize at least ten highly reactive B-cell epitopes in G1 and G2 (see Fig. 2 and Table 2), in comparison with the single epitope described for the recombinant G proteins of the closely related Sin Nombre virus (Jenison et al., 1994). Furthermore, we demonstrate the existence of antibodies against G1 and G2 in infected rodents (Fig. 2 and Table 2). These results differ from those obtained with immune sera from the rodent *Peromyscus maniculatus*, in which no reactivity against the *Escherichia coli* expressed G1 and G2 of Sin Nombre virus could be detected (Yamada et al., 1995). Among the 13 G epitopes identified in the present study, seven are recognized commonly by human and rodent immune sera. Furthermore, seven are predicted to be highly accessible while four comprise predicted immunogenic regions (data not shown). In addition, five of these epitopes are homologous to regions in PUUV and HTNV, which have been associated by others (Heiskanen et al., 1999; Hörling and Lundkvist, 1997; Koch et al., 2003; Wang et al., 1993) with the elicitation of neutralizing antibodies (Table 2). For ANDV N we found eight immunoreactive epitopes, of which five are recognized by humans and mice (Table 2). Of these epitopes, two are predicted to be highly accessible and two highly immunogenic (data not shown). The mayor antigenic determinants of ANDV N are different between humans and rodents, although both are located in the central region of the protein (see Fig. 1 and Table 2). On the other hand it has been reported that the mayor reactivity of immune sera to recombinant N produced in *E. coli* is directed dominantly to the amino terminal region (Gött et al., 1997; Jenison et al., 1994; Yamada et al., 1995).

Table 2
Average reactivity of human and rodent sera with peptides comprising the structural proteins of ANDV

Protein	Epitope (aa-aa)	Humans	Rodents
N	1-78		
	144-169		
	183-195		
	209-221		
	248-260		
	326-338		
	365-377		
	404-428		
G1	14-26		
	46-58		
	66-104		
	189-201		
	215-227		
	300-344		
	404-416		
	521-533		
	599-611		
G2	678-690		
	691-703		
	918-930		
	955-967		

White squares indicate no reactivity (average scoring grade <0.5), gray squares moderate reactivity (average scoring grade ≥0.5 and <1.5), and black squares strong reactivity (average scoring grade ≥1.5). Epitope regions marked in bold letters comprise amino acids homologous to regions involved in the neutralization of HTNV (Koch et al., 2003; Wang et al., 1993) and underlined epitopes include amino acids which are homologous to regions recognized by HTNV non-neutralizing monoclonal antibodies (Wang et al., 1993).

The discrepancy of our results obtained with synthetic peptides with data using recombinant truncated proteins might be explained by the possibility that linear epitopes might be hidden or not properly presented in *E. coli* expressed proteins, situation also described by other authors (Lundkvist et al., 1996). Since antibodies developed in infected individuals recognize mostly surface structures of the native proteins, they may still react with short linear peptides that represent only parts of a conformational epitope (Davies et al., 1990). Hence, immunoreactive regions recognized by sera with the pepscan approach may contribute to the identification of at least parts of sites for antibody binding, providing valuable comprehension about the surface topology and antigenicity of the structural proteins of ANDV. This insight affords important information for the future design of neutralizing monoclonal antibodies, a powerful tool in the development of immunotherapies for the acute phase of HCPS.

Materials and methods

Patient and rodent sera

Sera samples from Chilean patients with confirmed HCPS (n = 23) were provided by the Instituto de Salud Pública de Chile. Blood samples from naturally infected rodents (n = 7) and negative controls were obtained from Dr. Rebecca Aldunate from the Pontificia Universidad Católica. Human sera from healthy individuals used as negative controls were obtained from the Hospital Salvador of Santiago.

ELISA

Microtiter plate wells (Polysorb, Nunc, Rochester, USA) were activated with 100 ng of recombinant nucleoprotein. After 1 h incubation, the wells were blocked with 2% casein sacarose for 1 h at 37 °C. Then 50 μl of sera in dilutions of 1:400 to 1:6400 was applied to the wells and incubated for 1.5 h at 22 °C. Each serum sample was tested in duplicate. As second antibody 50 μl of goat anti-human IgG horseradish peroxidase (HRP) conjugate or goat anti-human IgM HRP conjugate (KPL, Maryland, USA) was added in 1:2000 dilutions and incubated for 30 min at 22 °C. After

a 30-min incubation with 50 μl of tetramethylbenzidine substrate (KPL, Maryland, USA), the reaction was stopped by the addition of 50 μl 3 N H_2SO_4. The optical density was measured at 450 nm.

Focus reduction neutralization assay (FRNT)

FRNT was performed as previously reported (Bharadwaj et al., 2000). 4.5×10^4 Vero E6 cells were seeded into each well of 48-well plates. The following day, 50–100 PFU of ANDV strain CHI-7913 (Galeno et al., 2002) were incubated with varying dilutions of heat-inactivated test serum. After 1 h at 37 °C, the cell culture media were replaced with the pre-incubated virus–serum mixture. After 4 h at 37 °C the virus and sera containing medium was removed and substituted with fresh media containing 1.2% methylcellulose. After 7 days the cells were fixed with 100% methanol containing 0.5% H_2O_2. Rabbit anti-nucleoprotein serum (1:5000) was added and incubated for 1 h at 37 °C. For detection, a goat anti-rabbit IgG (H + L) HRP conjugate in a 1:1000 dilution (Jackson Immuno-Research, West Grove, PA, USA) was added and after 1 h developed with a DAB/metal concentrate substrate (Pierce, Rockford, IL, USA). Finally, the number of viral foci were counted under an inverted microscope.

Epitope mapping

Linear B-cell epitopes were identified by the use of the pepscan method (Geysen et al., 1987). Peptides comprising the whole nucleocapsid and glycoprotein precursor (GPC) sequences of the Chilean isolate CHI-7913, GenBank accession numbers AAO86636 and AY228238, respectively (Tischler et al., 2003), were synthesized (Sigma-Genosys, The Woodlands, USA). In total, 166 13-mers peptides covalently linked to nitrocellulose membranes were analyzed for their reactivity with sera samples as recommended by the manufacturer instructions. Briefly, previously blocked membranes were incubated with sera in dilutions of 1:200 to 1:1000 for 3 h at 22 °C. Second antibodies conjugated to HRP were used in dilutions of 1:2000 to 1:5000 and incubated for 1 h at 22 °C. The different second antibody conjugates were goat anti-human polyvalent immunoglobulins (G, A, M) HRP conjugate (Sigma, St. Louis, MO, USA) and goat anti-*Peromyscus leucopus* IgG (H + L) conjugated to HRP (KPL, Maryland, USA). Bound antibodies were detected with SuperSignal Ultra (Pierce, Rockford, IL, USA) and chemiluminescence measured by exposing the membranes 10–60 s to an X-ray film. The reactivity was classified in three relative intensity scales: no reactivity in white (grade 0), moderate reactivity in gray (grade 1), and strong reactivity in black squares (grade 2). A visible example of the grading of reactivity is shown in Supplemental Fig. 1. Epitopes were defined as immunodominant when the average score of human or mice sera was ≥grade 1.5.

Prediction of accessible and antigenic regions

Protein side chains relative solvent accessibility was predicted by the method of Mucchielli-Giorgi et al. (1999), http://bioserv.rpbs.jussieu.fr. Antigenic regions were predicted utilizing the Vector NTI 8.0 (Welling et al., 1985).

Acknowledgments

We thank Maite Valdivia for excellent technical assistance. Thanks also to Dr. Rebecca Aldunate and Dr. Marcela Ferrés of the Pontificia Universidad Católica and Dr. Constanza Castillo of the Universidad de la Frontera for providing sera samples. We thank Dr. Bernardita Mendez for a critical review of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2005.01.031.

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