

# Antibody Responses to Four Corners Hantavirus Infections in the Deer Mouse (*Peromyscus maniculatus*): Identification of an Immunodominant Region of the Viral Nucleocapsid Protein

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**Antibody responses to Four Corners hantavirus (FCV) infections in the deer mouse (*Peromyscus maniculatus*) were characterized by using FCV nucleocapsid protein (N), glycoprotein 1 (G1), and glycoprotein 2 (G2) recombinant polypeptides in Western immunoblot assays. Strong immunoglobulin G reactivities to FCV N were observed among FCV-infected wild *P. maniculatus* mice ( $n = 34$ ) and in laboratory-infected *P. maniculatus* mice ( $n = 11$ ). No immunoglobulin G antibody reactivities to FCV G1 or G2 linear determinants were detected. The strongest N responses were mapped to an amino-proximal segment between amino acids 17 and 59 (QLVTARQKLKDAERAVELDPDDVKNKSTLQSRRAAVSALETKLG). FCV N antibodies cross-reacted with recombinant N proteins encoded by Puumala, Seoul, and Hantaan viruses.**

A North American hantavirus was identified recently as the cause of a human disease called hantavirus pulmonary syndrome (5, 20). The hantavirus pulmonary syndrome hantavirus is closely related to Prospect Hill virus and Puumala virus (PUU) and is more distantly related to Hantaan virus (HTN) and Seoul virus (SEO) (9, 20, 25, 27). The deer mouse (*Peromyscus maniculatus*) is the usual rodent host of the hantavirus pulmonary syndrome hantavirus (3). Proposed names for the virus have included pulmonary syndrome hantavirus, Muerto Canyon virus, and Sin Nombre virus. A uniform nomenclature has not yet been accepted. We will refer to the agent by the commonly used name Four Corners virus (FCV).

Hantavirus genomes consist of three single-stranded RNA segments called large (L), medium (M), and small (S) that encode four viral proteins (6, 22–24). The viral transcriptase is encoded by the L segment, the envelope glycoproteins G1 and G2 are encoded by the M segment, and the nucleocapsid (N) protein is encoded by the S segment. The G1 and G2 coding sequences are present within a continuous open reading frame that is transcribed as a single mRNA (24, 25).

In this study, we characterized antibody responses to FCV infections in the deer mouse, *P. maniculatus*. Blood samples from FCV-infected wild *P. maniculatus* and laboratory-infected *P. maniculatus* mice were tested for immunoglobulin G (IgG) antibody reactivities. S and M segment cDNAs representing N, G1, and G2 protein coding sequences were expressed as recombinant proteins in *Escherichia coli*. Detergent-denatured recombinant proteins were used as antigen targets in Western immunoblot assays. Because the target antigens were denatured, the current analysis was restricted to the characterization of antibodies that react with continuous (linear) amino acid determinants. The locations of antigenic sites were

mapped. Also, the FCV N protein region recognized by a cross-reactive monoclonal antibody (MAb) generated against PUU 83-223L (GB04-BF07) (21) was determined.

**Expression plasmid constructs.** FCV cDNAs were generated from FCV-infected human lung tissue (case identifier number 3H226) by using the reverse transcriptase-PCR technique (9, 11). PUU expression plasmid pPUU-N was derived from PUU P360 cDNA (28) obtained from C. Schmaljohn, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. SEO N and HTN N cDNAs were generated from virus-infected Vero E6 cell cultures (ATCC C1008) by using the reverse transcriptase-PCR technique. SEO HR80-39 (15) was provided by H. W. Lee, World Health Organization Collaborating Centre for Virus Reference and Research (Haemorrhagic Fever with Renal Syndrome), Seoul, Korea. HTN 76-118 (16) was obtained from the American Type Culture Collection (ATCC VR-938). Hantavirus cDNAs were inserted into the expression plasmids pATH and pMAL-c2 and were expressed as fusion proteins in *E. coli* (7, 14, 19). pATH recombinant plasmids pFCV-S-1224, -1131, -330, and -752; pFCV-M-1275 and -2028; and pPUU-N have been described previously (11). Amino acid coordinates of the hantavirus-encoded recombinant polypeptides are displayed in Table 1 and Fig. 1A.

**Epitope mapping studies.** Antibody-reactive segments of the pFCV-S-330 protein were mapped by testing nested sets of carboxy-to-amino-terminus deletions and amino-to-carboxy-terminus deletions for antibody reactivities in the Western blot assay. The deletion constructs and the mapping strategy have been described previously (11, 13). The amino acid coordinates of selected deletion clones are displayed in Table 2 and Fig. 1B.

**Western blot assays.** Western blot assays were performed as described previously (12). *P. maniculatus* serum samples were incubated at a 1:1,000 dilution (10  $\mu$ l of serum in 10 ml of buffer) for 16 h with recombinant proteins fixed to nitrocellulose blots. Antigen-antibody complexes were detected by incubating the blots with alkaline phosphatase-conjugated goat an-

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TABLE 1. Hantavirus expression constructs

Virus	RNA segment (open reading frame)	Construct frame	Nucleotide coordinates <sup>a</sup>	Amino acid coordinates <sup>b</sup>	Expression vector	Lane no. <sup>c</sup>
FCV	S (N)	pFCV-N	43–1329	1–428	pMAL-c2	1, 9, 13
		pFCV-S-1224	43–1257	1–405	pATH HT-1	5
		pFCV-S-1131	123–1257	35–405	pATH21	6
		pFCV-S-752	509–1257	157–405	pATH1	7
		pFCV-S-330	43–378	1–112	pATH HT-1	8, 14
	M (G1)	pFCV-M-1275	136–1401	29–450	pATH23	2
	M (G1/G2)	pFCV-M-2028	1324–3333	425–1094	pATH23	3
	M (G2)	pFCV-M-543	3166–3696	1039–1140	pMAL-c2	4
PUU	S (N)	pPUU-N	43–1341	1–428	pATH10	10
SEO	S (N)	pSEO-N	43–1326	1–423	pMAL-c2	11
HTN	S (N)	pHTN-N	43–1326	1–423	pMAL-c2	12

<sup>a</sup> Numbered with respect to the homologous positions in the sequences of the pulmonary syndrome hantavirus S and M segments (GenBank accession numbers L25784 and L25783, respectively) (25).

<sup>b</sup> Numbered with respect to the homologous positions in the deduced amino acid sequences of the pulmonary syndrome hantavirus S and M open reading frames, counting the first methionine residue as position 1 (25).

<sup>c</sup> Lane number refers to the lanes in Fig. 2 and the numbers in black circles in Fig. 1.

ti-*Peromyscus leucopus* IgG antiserum (Kirkegaard & Perry Laboratories) at a 1:1,000 dilution for 4 h. Alkaline phosphatase activity was detected by incubating the blots for 10 min in alkaline buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Mouse MAbs were tested similarly and at a 1:1,000 dilution; the secondary antibody was goat anti-*Mus musculus* IgG (Boehringer Mannheim).

**FCV antibody responses among FCV-infected wild *P. maniculatus* mice.** Thirty-four hantavirus-infected wild *P. maniculatus* mice were tested for FCV antibody reactivities. *P. maniculatus* mice were trapped in the states of New Mexico ( $n = 10$ ), Arizona ( $n = 16$ ), and California ( $n = 8$ ) between June 1993 and June 1994. Lung tissue samples were tested for the presence of FCV genetic sequences by the reverse transcriptase-PCR technique and nucleotide sequence analysis as described previously (8, 9).

Blood samples from 34 of 34 FCV-infected wild *P. maniculatus* mice contained strong IgG antibody reactivities to FCV N protein. All blood samples that reacted with the full-length FCV N protein pFCV-N (amino acids [aa] 1 to 428) also reacted with the pFCV-S-1224 (aa 1 to 405) and pFCV-S-330 (aa 1 to 112) proteins (Fig. 2, panels 2A and 3A, lanes 1, 5, and 8). In 3 of the 34 samples, weak reactivities to the pFCV-S-1131 (aa 35 to 405) and pFCV-S-752 (aa 157 to 405) proteins were also seen (Fig. 2, panel 3A, lanes 6 and 7). In the remaining 31 samples, no reactivities to these proteins were detected. These findings localized the major linear epitope (or epitopes) to the amino-proximal 112 amino acids of FCV N protein.

The FCV N polypeptide segment that contains the major antibody-reactive region was defined further by deletion mapping. Nested sets of amino-to-carboxy-terminus deletions and carboxy-to-amino-terminus deletions were constructed in the pFCV-S-330 protein (11). The nested sets of deleted proteins were tested for antibody reactivities in Western blot assays. *P. maniculatus* IgG antibodies reacted strongly with the pFCV-S-330 protein (aa 1 to 112) and reacted substantially less strongly with the pFCV-S-NEx91 protein (aa 17 to 112) (Fig. 2, panels 2B and 3B, lanes 14 and 15). There was no reactivity or minimal reactivity with the pFCV-S-NEx136 protein (aa 32 to 112) and with proteins that contained more extensive amino-terminus deletions (Fig. 2, panels 2B and 3B, lanes 16 to 18). Therefore, the amino-terminus boundary of the antibody-reactive region lies carboxy-proximal to aa 17. However, inclusion of the segment from aa 1 to 17 substantially increased antibody reactivity. Antibodies reacted strongly with the carboxy-terminus deletions up to aa 59 (pFCV-S-CEx220) (Fig. 2, panels 2B and 3B, lanes 19 to 23). There was no detectable reactivity with the pFCV-S-CEx170 protein (aa 1 to 41) and proteins that contained more extensive carboxy-terminus deletions (Fig. 2, panels 2B and 3B, lanes 24 and 25). These findings localized the carboxy-terminus boundary of the antibody-reactive region amino proximal to aa 59. Therefore, the FCV N polypeptide segment that reacted most strongly with *P. maniculatus* IgG antibodies was localized to the segment between aa 17 and 59. However, the reactivity was substantially stronger when the segment included aa 1 to 59. This pattern of antibody reactivity was observed with all of the blood samples from the 34 FCV-infected wild *P. maniculatus* mice.

All 34 FCV N protein-reactive blood samples cross-reacted with the PUU N recombinant protein pPUU-N. The intensity of PUU N protein antibody staining varied among the mice tested. In some cases, the PUU N protein antibody staining was of similar intensity to the staining of FCV N protein. In other cases, the PUU N protein antibody staining was substantially less intense than that of FCV N protein (Fig. 2, panels 2A and 3A, lanes 9 and 10). Thirteen samples also cross-reacted with the SEO N and HTN N recombinant proteins, but the antibody staining was weak in all cases (Fig. 2, panel 3A, lanes 11 and 12). In the remaining 21 samples, SEO N and HTN N protein reactivities were not detected.

TABLE 2. Epitope mapping deletion constructs<sup>a</sup>

Construct name	Nucleotide coordinate <sup>b</sup>		Amino acid coordinate <sup>b</sup>		Lane no. <sup>c</sup>
	5'	3'	Amino	Carboxy	
pFCV-S-330	43	378	1	112	8, 14
pFCV-S-NEx91	91	378	17	112	15
pFCV-S-NEx136	136	378	32	112	16
pFCV-S-NEx181	181	378	47	112	17
pFCV-S-NEx220	220	378	60	112	18
pFCV-S-CEx246	43	246	1	68	22
pFCV-S-CEx220	43	220	1	59	23
pFCV-S-CEx170	43	170	1	41	24
pFCV-S-CEx125	43	125	1	27	25

<sup>a</sup> The N open reading frame was used throughout.

<sup>b</sup> Numbered with respect to the homologous positions in the sequences of the pulmonary syndrome hantavirus (25).

<sup>c</sup> Lane number refers to the lanes in Fig. 2 and the numbers in black circles in Fig. 1.

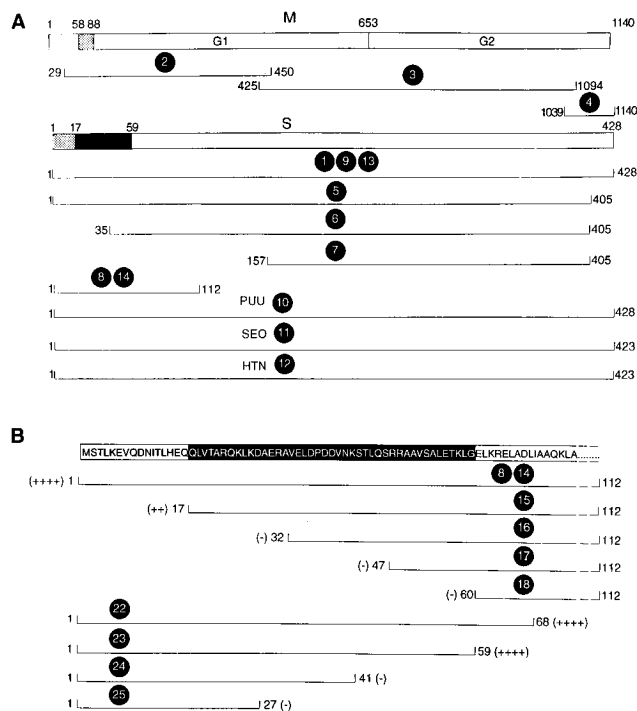


FIG. 1. Maps of hantavirus recombinant proteins. (A) FCV M and S segment polypeptides are represented as rectangular boxes. Numbers above the boxes refer to amino acid coordinates (25). The marker at M segment aa 653 is the probable boundary between the G1 and G2 polypeptides (24, 25). The cross-hatched segment between aa 58 and 88 marks a G1 region recognized by human FCV antibodies. The S segment block from aa 17 to 59 marks the immunodominant region recognized by rodent and human FCV N protein antibodies. The stippled segment from aa 1 to 17 is the N-terminal region, which, when deleted from the aa 1-to-aa 59 segment, substantially decreases but does not eliminate antibody reactivities. Bars beneath the boxes represent recombinant polypeptides. Numbers at the ends of the bars represent amino acid coordinates of the amino terminus and carboxy terminus, respectively. Bars marked PUU, SEO, and HTN represent N recombinant proteins encoded by those viruses. Numbers in black circles represent the lane positions of the recombinant proteins in Fig. 2. The rectangular box in panel B is an expanded view of the immunodominant FCV N segment (aa 1 to 59). Letters within the box represent the amino acid sequence. The bars beneath the box represent the FCV N protein deletion constructs. The (+) and (-) symbols refer to the intensity of antibody reactivity.

No IgG antibody reactivities to the FCV G1 and FCV G2 recombinant proteins were detected in any of the 34 *P. maniculatus* blood samples tested (Fig. 2, panels 2A and 3A, lanes 2 to 4).

**FCV antibody responses among laboratory-infected *P. maniculatus* mice.** Seventeen *P. maniculatus* mice that were experimentally inoculated with FCV were tested for FCV antibody reactivities. Blood samples were obtained from P. Rollin, Centers for Disease Control and Prevention, Atlanta, Ga. Thirteen mice received intraperitoneal injections of FCV-infected *P. maniculatus* lung tissue; 4 mice received intraperitoneal injections of Vero E6-adapted FCV. One blood sample from each animal was tested. Samples were collected at various times after inoculation, including days 14 ( $n = 4$ ), 28 ( $n = 6$ ), 37 ( $n = 2$ ), 39 ( $n = 1$ ), and 65 ( $n = 4$ ). The samples collected at day 65 postinoculation were from the mice that had received injections of Vero E6-adapted FCV. Two of four day 14 samples, one of six day 28 samples, and all seven samples collected on or after day 37 contained strong FCV N protein IgG antibody responses (Fig. 2, panel 1A). None of the blood samples contained detectable IgG antibody reactivities to the FCV G1 and FCV G2 recombinant proteins.

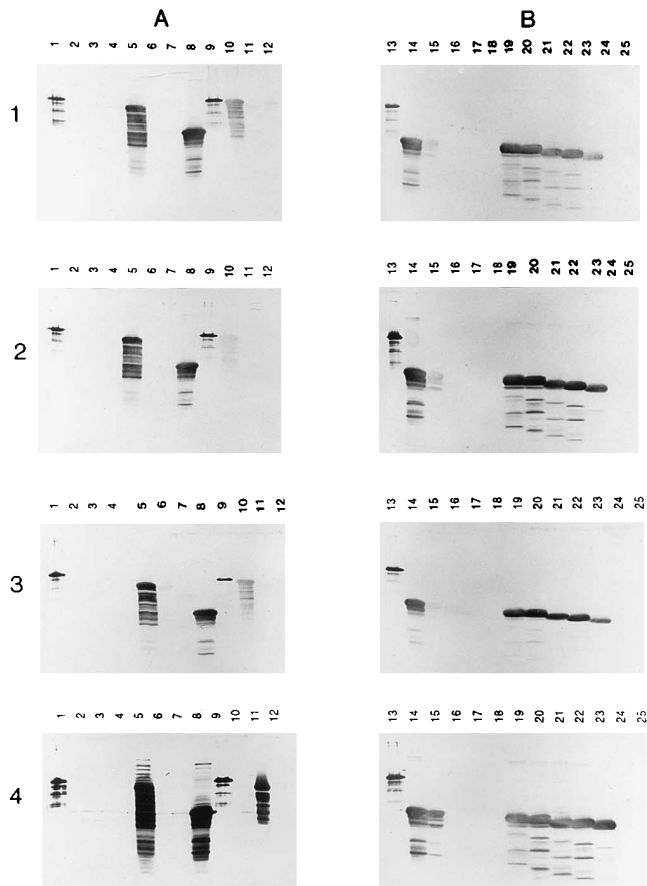


FIG. 2. Recombinant Western blot assays. Panels A and B are replicate Western blots. Blots were reacted with blood samples from laboratory-infected *P. maniculatus* mice (row 1), wild FCV-infected *P. maniculatus* mice (rows 2 and 3), and MAB GB04-BF07 (row 4). Lane positions correspond to the numbers in black circles in Fig. 1.

The 10 samples that reacted with pFCV-N protein (aa 1 to 428) also reacted with the pFCV-S-1224 (aa 1 to 405) and pFCV-S-330 (aa 1 to 112) proteins (Fig. 2, panel 1A, lanes 1, 5, and 8). One sample reacted weakly with the pFCV-S-1131 (aa 35 to 405) and pFCV-S-752 (aa 157 to 405) proteins; the remaining nine samples did not contain detectable antibodies to these proteins. In all 10 samples tested, the FCV N protein IgG antibody reactivities mapped to the same region that was recognized by IgG antibodies from FCV-infected wild *P. maniculatus* mice (Fig. 2, panel 1B). All 10 FCV N protein-reactive samples cross-reacted with the PUU N recombinant protein. Seven samples also reacted weakly with the SEO N and HTN N proteins.

**Comparisons of human and *P. maniculatus* antibody responses to FCV infections.** We have shown previously that human FCV infections elicit both N and G1 protein antibody responses (11). Human FCV N protein antibody responses cross-react strongly with PUU N and PHV N proteins, but human FCV G1 protein antibody responses are type specific relative to PUU G1 and PHV G1 proteins. The dominant region recognized by human FCV N protein antibodies (aa 17 to 59) is the same region that is recognized most strongly by *P. maniculatus* FCV N protein antibodies. This region was immunodominant both in acutely infected (laboratory-infected) mice and in wild-caught *P. maniculatus* mice that are presumed

to be chronically infected with FCV. *P. maniculatus* FCV N protein antibodies also cross-reacted with N proteins of related hantaviruses.

No IgG antibody reactivities to FCV G1 or G2 recombinant proteins were detected in blood samples from FCV-infected *P. maniculatus* mice. In contrast, human FCV infections elicit strong G1 protein antibody responses that are directed against an amino-proximal segment between aa 58 and 88 (11). The M segment recombinant proteins that were used in this study included all of the G1 and G2 polypeptides, except for the amino-terminal 28 aa of G1. Therefore, it is possible that FCV infection in *P. maniculatus* mice elicits G1 antibodies that are dependent upon this amino-terminal segment for binding.

Compared with the type-specific serodiagnosis of human FCV infections that is possible because of FCV G1 protein antibodies, type-specific hantavirus serodiagnosis in rodents may prove to be more problematic. We have tested serum samples from eight western harvest mice (*Reithrodontomys megalotis*) for antibody reactivities to FCV recombinant proteins (8, 10). These western harvest mice were infected with a newly identified hantavirus that is enzootic in *R. megalotis* animals and that we will refer to as harvest mouse virus (HMV) (8). Genetic sequence analysis suggests that HMV is closely related to FCV. The nucleotide sequence distance and the amino acid sequence distance between HMV and FCV are 23.7 and 15%, respectively (9). *R. megalotis* HMV antibodies reacted with the same FCV N segment that is recognized by *P. maniculatus* FCV antibodies. In all cases, the reactivities of *R. megalotis* HMV antibodies with FCV recombinant proteins were not distinguishable from the reactivities of *P. maniculatus* FCV antibodies. Therefore, the type-specific diagnosis of closely related hantavirus infections in rodents is complicated by the cross-reactivity of antibodies generated against the immunodominant amino-terminal N segment. In rodents, definitive type-specific hantavirus identification may require nucleotide sequence analysis of viral cDNAs (1, 3, 8).

**Reactivity of PUU MAb GB04-BF07 with hantavirus recombinant proteins.** The location of the cross-reactive epitope recognized by PUU MAb GB04-BF07 was mapped. MAbs GB04-BF07 and DA03-BA07 were obtained from C. J. Peters, Centers for Disease Control and Prevention. GB04-BF07 was generated against PUU 83-223L (21) and is known to react with the PUU 83-223L N protein. The reactivities of GB04-BF07 with whole viral lysates of HTN, SEO, PHV, and PUU in the enzyme immunoassay have been described previously by Ruo et al. (21). In the enzyme immunoassay, GB04-BF07 reacts strongly with PUU 83-223L, cross-reacts strongly with Prospect Hill virus and SEO, and cross-reacts weakly with HTN. GB04-BF07 was tested in a Western blot assay with N recombinant proteins encoded by FCV, PUU P360, SEO HR80-39, and HTN 76-118 (Fig. 2, panel 4A). GB04-BF07 reacted strongly with FCV N and SEO N proteins, weakly with HTN N protein, and not at all with PUU P360 N protein. DA03-BA07, which also was generated against PUU, reacted strongly with PUU P360 N protein, weakly with SEO N and HTN N proteins, and not at all with FCV N protein (data not shown). The reactivity of GB04-BF07 with FCV N protein was mapped to the segment between aa 17 and 59, which is the same segment that was recognized by *P. maniculatus* FCV N protein antibodies (Fig. 2, panel 4B).

PUU infects the bank vole (*Clethrionomys glareolus*) and is endemic to northern Europe (2). Phylogenetic analysis indicates that Scandinavian PUU strains (83-223L, Sotkamo, and Hällnäs-B1) are diverged from Russian PUU strains (P360, CG18-20, and K27) but that PUU strains are more closely related to one another than to FCV, SEO, and HTN strains

(26–28). Our data indicate that the immunodominant N segment between aa 17 and 59 is sufficiently conserved to result in strong GB04-BF07 cross-reactivity with FCV N and SEO N proteins. However, Russian PUU P360 N protein showed no cross-reactivity with GB04-BF07, which was generated against Scandinavian PUU 83-223L. Therefore, type-specific serodiagnosis of hantavirus infections in rodents may also be complicated by antigenic variability within the immunodominant region between closely related virus strains (4, 17, 18).

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