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Characterization of Human Antibody Responses to Four Corners Hantavirus Infections among Patients with Hantavirus Pulmonary Syndrome

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Hantavirus pulmonary syndrome (HPS) is a human disease caused by a newly identified hantavirus, which we will refer to as Four Corners virus (FCV). FCV is related most closely to Puumala virus (PUU) and to Prospect Hill virus (PHV). Twenty-five acute HPS serum samples were tested for immunoglobulin G (IgG) and IgM antibody reactivities to FCV-encoded recombinant proteins in Western blot (immunoblot) assays. All HPS serum samples contained both IgG and IgM antibodies to the FCV nucleocapsid (N) protein. FCV N antibodies cross-reacted with PUU N and PHV N proteins. A dominant FCV N epitope was mapped to the segment between amino acids 17 and 59 (QLVTARQKLKDAERAVELDPDDVNKSTLQSRRAAVSALETKLG). All HPS serum samples contained IgG antibodies to the FCV glycoprotein-1 (G1) protein, and 21 of 25 serum samples contained FCV G1 IgM antibodies. The FCV G1 antibodies did not cross-react with PUU G1 and PHV G1 proteins. The FCV G1 type-specific antibody reactivity mapped to a segment between amino acids 59 and 89 (LKIESSCNFDLHVPATTTQKYNQVDWTKKSS). One hundred twenty-eight control serum samples were tested for IgG reactivities to the FCV N and G1 proteins. Nine (7.0%) contained FCV N reactivities, 3 (2.3%) contained FCV G1 reactivities, and one (0.8%) contained both FCV N and FCV G1 reactivities. The epitopes recognized by antibodies present in control serum samples were different from the epitopes recognized by HPS antibodies, suggesting that the control antibody reactivities were unrelated to FCV infections. These reagents constitute a type-specific assay for FCV antibodies.

A newly discovered hantavirus has been implicated in a severe respiratory disease in North America. The hantavirus pulmonary syndrome (HPS) was first recognized among cases that occurred in the Four Corners area (the region surrounding the common border of New Mexico, Arizona, Utah, and Colorado) in 1993 (3–6). As of 30 November 1993, 48 cases had been reported, with a case fatality rate of 56%. HPS is characterized by rapidly progressive pulmonary edema, thrombocytopenia, and, preterminally, refractory systemic hypotension. Respiratory symptoms often are preceded by a prodrome of fever and severe myalgias. Patients usually have sought medical attention because of progressive dyspnea. Characteristic clinical findings at presentation have included hypoxemia, thrombocytopenia, hemoconcentration, hypoproteinemia, leukocytosis, and diffuse bilateral interstitial pulmonary infiltrates on chest radiograms. Abnormalities at autopsy have included alveolar and interstitial pulmonary edema, pleural effusions, and lymphoid cell infiltration of the pulmonary interstitium.

In June 1993, the Centers for Disease Control and Prevention (CDC) reported that HPS patient serum samples contained immunoglobulin M (IgM) antibodies that reacted with the hantaviruses Hantaan, Seoul, and Puumala (PUU) (3). Consensus oligonucleotide primers were used to amplify a hantavirus nucleic acid segment from lung tissue of a fatal human case. Sequence analysis indicated that the HPS agent was a previously uncharacterized hantavirus most closely related to PUU virus and to Prospect Hill virus (PHV) (10, 17). PUU is endemic in northern Europe and usually infects the bank vole *Clethrionomys glareolus*. Human infections cause a mild form of the hemorrhagic fever with renal syndrome called nephropathia epidemica. PHV first was isolated in Maryland from the meadow vole *Microtus pennsylvanicus*. Human infection has not been described. The HPS agent commonly has been called Four Corners virus and hantavirus pulmonary syndrome virus, but a uniform nomenclature for the HPS agents has not yet been accepted. We will refer to the agent by the commonly used name Four Corners virus (FCV). The predominant rodent host of FCV is the deer mouse *Peromyscus maniculatus* (6).

Hantaviruses are members of the family *Bunyaviridae*. Hantavirus genomes consist of three single-stranded RNA segments called large (L), medium (M), and small (S), respectively (22). Four viral proteins are encoded by virus-complementary RNAs (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. In human PUU infections, N and G1 antibodies appear during the acute phase of illness. G2 antibodies do not appear until the late convalescent period (8). In general, N antibody responses induced by one hantavirus cross-react with N proteins of other hantaviruses. N antibodies do not neutralize virus infectivity in vitro and do not protect against infection in experimental animal models. In contrast, G1 and G2 antibody responses include type-specific antibodies that neu-

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tralize virus in vitro and that are protective against infection in vivo (1, 2, 7, 13, 15, 16, 20, 21, 25–30).

To develop a serologic test for the diagnosis of FCV infections, we generated FCV-encoded recombinant polypeptides representing segments of the N, G1, and G2 proteins. HPS serum samples were tested for FCV IgG and IgM antibody reactivities to the recombinant proteins in Western blot (immunoblot) assays. The type specificity of FCV antibodies was assessed by testing for reactivity with homologous polypeptides encoded by PUU and PHV. The locations of dominant FCV G1 and N epitopes were mapped.

MATERIALS AND METHODS

Selection of human subjects. Serum samples were obtained from 25 HPS patients who were diagnosed between 14 May 1993 and 27 November 1993. All subjects have been reported as confirmed HPS cases by CDC. In the context of the characteristic acute respiratory disease, HPS was diagnosed by detecting hantavirus antibodies in serum and/or by detecting FCV nucleic acid sequences in tissue samples. Hantavirus antibody testing at CDC included enzyme-linked immunoassays to detect serum IgG and IgM antibody reactivities to Hantaan virus, Seoul virus, PUU, and PHV lysates (3). Detection of FCV nucleic acid sequences was performed at CDC and at the University of New Mexico by using the reverse transcriptase (RT)-PCR technique with hantavirus consensus primers (10, 17). FCV sequences were assayed in tissue samples obtained at autopsy or in peripheral blood leukocytes from living subjects (9a, 17). All subjects had resided in New Mexico (14 subjects), Arizona (9 subjects), or Colorado (2 subjects) at the time of disease onset. Serum samples were obtained at autopsy in seven cases. In the other 18 cases, serum samples were obtained at the time of hospitalization for respiratory insufficiency. Of the 25 subjects, 11 died with HPS and 14 survived the illness. Of the 14 subjects who survived, 4 had received mechanical ventilation during hospitalization.

Serum samples were obtained from 128 control subjects between 2 February 1993 and 26 August 1993. Control subjects had presented for anonymous human immunodeficiency virus antibody testing at a U.S. Public Health Service Hospital in Shiprock, N.Mex. Control subjects predominantly were pregnant women; 121 subjects (94.5%) were women, and 7 subjects (5.5%) were men. The average age of control subjects was 25.6 years (range, 15 to 72 years). One hundred eighteen subjects (92%) were Native American, eight subjects (6%) were Caucasian, and two subjects (2%) were of unknown ethnicity. State of residence was New Mexico for 109 subjects (85%), Arizona for 11 subjects (9%), Colorado for 3 subjects (2%), Utah for 3 subjects (2%), and unknown for 2 subjects (2%).

Lung tissue was obtained at autopsy from a patient who died with HPS (unique identifier number 3H226). Patient 3H226 had been a resident of New Mexico.

Expression plasmid constructs. Bacterial expression plasmids that contained FCV, PHV, and PUU strain P360 cDNAs were constructed. All FCV cDNAs were generated from virus-infected human lung tissue (patient-case 3H226) by using the RT-PCR technique (10). FCV cDNAs S-1131, M-1275, and M-2028 have been described previously (10). The nucleotide sequences of the S-1131 and M-1275 cDNAs are available from GenBank under accession numbers U02474 and U02471, respectively. S-419 was generated from lung tissue RNA by using a hantavirus S segment 5′-terminus consensus primer (5′-TAGTAGACTTCGT[A/G]AA[A/G]AGCTAC TA-3′) (22) and an FCV-specific antisense-strand primer (5′-GGATAATCGGTAATGCAAAACT-3′) (10). S-1224 was constructed by ligating S-330 and S-909 cDNAs by means of *Xho*I restriction enzyme sites present within their antisense- and sense-strand PCR primers, respectively. S-330 cDNA was generated from S-419 cDNA by PCR using FCV-specific primers. The sense-strand primer was 5′-TACGACTAAGC TTATGACGACCCTCAAAGAAG-3′, and the antisense-strand primer was 5′-TGGTTCCTCGAGGTCAATGGAATT TACATCAAG-3′. The underlined nucleotides are different from the FCV 3H226 sequence (5′-TTC-TAG-3′) in order to create an *Xho*I restriction enzyme recognition site; the nucleotide changes do not alter the FCV 3H226 N amino acid coding sequence. S-909 was generated from S-1131 DNA by PCR using an FCV-specific sense-strand primer (5′-ATT GACCTCGAGGAACCAAGTGGGCAAACAG-3′; underlined sequence is homologous to the *Xho*I recognition sequence in the antisense-strand primer of S-330) and the hantavirus antisense consensus primer that was used to generate S-1131 (5′-GGCTTCTAGAGGGATCCATGTCATC ACC-3′) (10). S-752 is a 752-bp fragment generated by digesting S-1131 with *Eco*RI. PHV cDNAs were generated from PHV-infected Vero E6 cell cultures (ATCC C1008) by using the RT-PCR technique. PHV S-1131 and M-1275 cDNAs were synthesized by using the same hantavirus consensus primers that were used to synthesize FCV S-1131 and M-1275, respectively (10). PHV S-330 was synthesized by using the same antisense consensus primer that was used to synthesize FCV S-330 (see above). The sense-strand primer was PHV specific and had the sequence 5′-TACTACAGTCGACGGGATGA GCCAACTCAGGGA-3′. PUU N and G1 recombinant DNAs were generated by PCR from plasmids that contained complete cDNAs of PUU strain P360 S and M segments, respectively, using P360-specific primers (N sense, 5′-CCATGGG TGACTTGACAGACATCCAAG-3′; N antisense, 5′-AGAT CTTATCTAAGGGCTTGGTT-3′; G1 sense, 5′-CCATGGG AGAACTTAGTCCAGTT-3′; G1 antisense, 5′-AGATCTG CACCCTTGCATAGGCTCATA-3′). PHV was provided by R. Yanagihara, National Institutes of Health, Bethesda, Md. PUU strain P360 cDNAs contained in bacterial plasmid vectors were provided by C. Schmaljohn, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. PHV and PUU nucleotide sequences were obtained from the National Center for Biotechnology Information (GenBank accession numbers L11347, L08755, X55129, and X55128) (18, 19).

Hantavirus cDNA-encoded polypeptides were expressed in *Escherichia coli* HB101 by means of pATH plasmid expression vectors (14). Expression vectors pATH1, pATH10, pATH11, pATH20, pATH21, pATH22, and pATH23 were obtained from the American Type Culture Collection (ATCC 37695 through 37703, respectively). pATH HT-1 was constructed from pATH23 by substituting the polylinker segment between the *Xba*I and *Kpn*I sites with the double-stranded oligonucleotide 5′-ACATCACCATCACCATCACCTCGAG-3′. pATH vectors contain 5′ transcription control elements and a portion of the first structural gene (*trpE*) of the *E. coli* tryptophan synthetase operon. Hantavirus cDNA segments were inserted into pATH DNA at unique restriction enzyme sites within a polylinker segment located 3′ to the *trpE* gene. Hantavirus cDNA-encoded polypeptides were expressed as fusion proteins linked to a 37,000-Da polypeptide encoded by *trpE*. Nucleotide coordinates of the FCV, PHV, and PUU cDNA segments that were used to express hantavirus recombinant polypeptides are displayed in Table 1. Nucleotide and amino acid coordinates for FCV and PUU are expressed relative to the corresponding coordinates of PHV (18, 19).

Synthesis of fusion proteins, SDS-polyacrylamide gel elec-

TABLE 1. Hantavirus expression constructs

Virus	RNA segment (open reading frame)	Construct name	Nucleotide coordinates[a]	Amino acid coordinates[b]	Expression vector	Restriction sites
FCV	S (N)	pFCV-S-1224	43–1266	1–408	pATH HT-1	*Hin*dIII-*Xba*I
		pFCV-S-1131	166–1266	41–408	pATH21	*Pst*I-*Kpn*I
		pFCV-S-330	43–372	1–110	pATH HT-1	*Hin*dIII-*Xho*I
		pFCV-S-752	509–1266	157–408	pATH1	*Eco*RI-*Eco*RI
	M (G1)	pFCV-M-1275	141–1407	32–452	pATH23	*Hin*dIII-*Xba*I
	M (G1/G2)	pFCV-M-2028	1327–3355	427–1102	pATH23	*Pst*I-*Kpn*I
PUU	S (N)	pPUU-N	43–1341	1–433	pATH10	*Eco*RI-*Bam*HI
	M (G1)	pPUU-G1	50–1909	1–620	pATH10	*Eco*RI-*Bam*HI
PHV	S (N)	pPHV-S-1131	166–1266	41–408	pATH21	*Pst*I-*Kpn*I
		pPHV-S-330	43–372	1–110	pATH HT-1	*Hin*dIII-*Xho*I
	M (G1)	pPHV-M-1275	141–1407	32–452	pATH1	*Eco*RI-*Eco*RI

[a] Numbered with respect to the homologous positions in the sequences of PHV S and M segments (see references 18 and 19, respectively).
[b] Numbered with respect to the homologous positions in the open reading frames of PHV S and M segments, counting the first methionine codon as position 1 (see references 18 and 19, respectively).

trophoresis, and Western immunoblot assays. The expression of fusion proteins in *E. coli*, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and Western immunoblot assays were performed as described previously (11). In assays to detect the presence of hantavirus antibodies, the bacterial fusion proteins were partially purified from *E. coli* proteins (14). In epitope mapping studies, whole bacterial lysates were used as antigen targets. Human serum samples were incubated with Western blots at a 1:200 dilution for 16 h at 4°C. Antigen-antibody complexes were detected by incubating the Western blots with alkaline phosphatase-conjugated goat anti-human IgG or goat anti-human IgM antisera (Boehringer Mannheim) at a 1:1,000 dilution for 4 h at room temperature. 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 (11).

Epitope mapping studies. Antibody-reactive regions of the recombinant proteins were mapped by generating nested sets of deletions in the FCV DNA segments as described previously (12). Unidirectional deletions were generated in the FCV cDNA inserts of recombinant plasmids by using exonuclease III and nuclease S1 as described by Henikoff (9). To prepare the 3′-to-5′ deletions in M-1275, pFCV-M-1275 was digested with *Not*I and *Nsi*I. To prepare the 5′-to-3′ deletions in M-1275, M-1275 cDNA in plasmid vector pCRII was digested with *Kpn*I and *Spe*I; selected deletion clones were digested with *Hin*dIII and *Eco*RI, and the FCV cDNA-containing fragments were inserted into pATH23 (*Hin*dIII-*Eco*RI digest). To prepare the 3′-to-5′ deletions in S-330, pFCV-S-330 was digested with *Xho*I and *Sst*I. To create the 5′-to-3′ deletions in S-330, site-specific deletions were made by generating PCR amplicons with FCV-specific oligonucleotide primers. The deleted plasmids expressed nested series of truncated recombinant proteins that were reacted with human serum antibodies in Western blot assays. DNA sequence analysis was used to determine the nucleotide coordinates of deletion clones that defined the boundaries of immunoreactive regions. Nucleotide coordinates and amino acid coordinates of the deletion clones are displayed in Table 2.

RESULTS

Serum FCV IgG and IgM antibody reactivities in HPS patients. Serum samples from 25 HPS patients were tested for IgG and IgM antibody reactivities to FCV, PHV, and PUU recombinant proteins. All subjects had resided in New Mexico, Arizona, or Colorado at the time of disease onset. Serum samples were obtained at autopsy in seven cases. For the other 18 cases, serum samples were obtained at the time of hospitalization for signs of respiratory insufficiency. The FCV recombinant proteins were encoded by cDNAs derived from FCV-infected lung tissue. The infected lung tissue was obtained at autopsy from a New Mexico resident who died with HPS (10).

Serum antibodies were reacted with hantavirus-encoded recombinant proteins in Western immunoblot assays. The FCV, PHV, and PUU cDNA-containing expression constructs are shown in Table 1. Antibodies reacted with the full-length hantavirus-encoded fusion proteins and with multiple smaller-molecular-weight proteins (Fig. 1). The smaller-molecular-weight bands are observed commonly with TrpE fusion proteins and probably represent degradation products that retain the antibody-reactive epitope (12). Serum samples were tested at a dilution of 1:200 (50 μl of serum diluted in 10 ml of buffer). Antibody reactivities were graded as 0 (no staining) to 4+ (intense staining). Antibody titers were determined by terminal dilution for 10 HPS serum samples that contained reactivities to the FCV N protein encoded by pFCV-S-330. Antibody staining intensities of 2+, 3+, and 4+ at a serum dilution of

TABLE 2. Epitope mapping deletion constructs

Open reading frame	Construct name	Coordinate[a]			
		Nucleotide		Amino acid	
		5′	3′	Amino	Carboxy
G1	pFCV-M-1275	141	1407	32	452
	pFCV-M-NEx222	222	1407	59	452
	pFCV-M-NEx297	297	1407	84	452
	pFCV-M-CEx372	141	372	32	107
	pFCV-M-CEx316	141	316	32	89
	pFCV-M-CEx237	141	237	32	62
N	pFCV-S-330	43	372	1	110
	pFCV-S-NEx91	91	372	17	110
	pFCV-S-NEx136	136	372	32	110
	pFCV-S-NEx181	181	372	47	110
	pFCV-S-NEx220	220	372	60	110
	pFCV-S-CEx246	43	246	1	68
	pFCV-S-CEx220	43	220	1	59
	pFCV-S-CEx170	43	170	1	41
	pFCV-S-CEx125	43	125	1	27

[a] Numbered with respect to the homologous positions in the sequences of PHV S and M segments (see references 18 and 19, respectively).

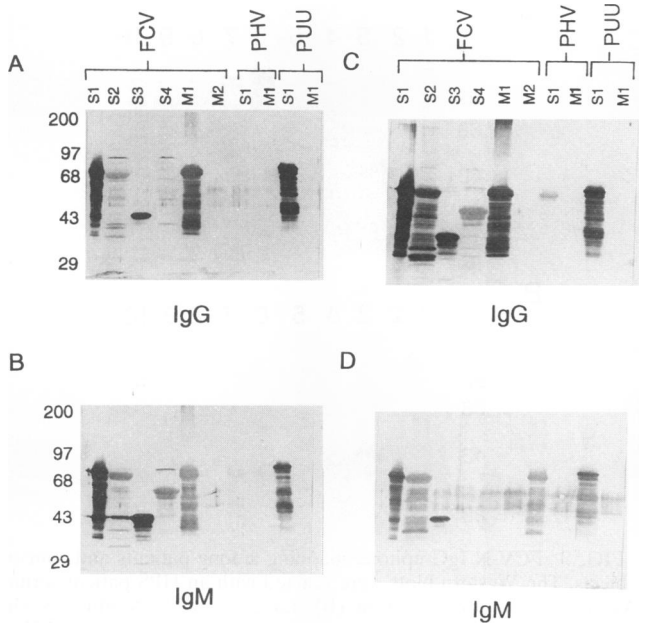

FIG. 1. Hantavirus Western immunoblot assays. (A and B) Recombinant proteins reacted with serum from one HPS patient; (C and D) recombinant proteins reacted with serum from a second HPS patient; (A and C) detection of IgG antibodies; (B and D) detection of IgM antibodies. Lanes contain recombinant bacterial proteins encoded by FCV-S-1224 (FCV S1), pFCV-S-1131 (FCV S2), pFCV-S-330 (FCV S3), pFCV-S-752 (FCV S4), pFCV-M-1275 (FCV M1), pFCV-M-2028 (FCV M2) pPHV-S-1131 (PHV S1), pPHV-M-1275 (PHV M1), pPUU-N (PUU S1), and pPUU-G1 (PUU M1). See Table 1 for a detailed description of the expression constructs. Numbers at the left mark the mobilities of protein molecular mass standards in kilodaltons.

1:200 were associated with endpoint dilution titers of approximately 1:1,000, 1:10,000, and 1:100,000, respectively.

All HPS serum samples contained IgG antibodies that reacted with the FCV N protein and with the FCV G1 recombinant protein (Table 3). In general, FCV N IgG antibody reactivities were stronger than FCV G1 IgG antibody reactivities. Forty-four percent of the FCV N IgG reactivities were graded as 4+, and 48% of the FCV G1 IgG reactivities were graded as 2+. All HPS serum samples contained IgM antibodies that reacted with the FCV N protein. For an individual subject, the intensities of the FCV N IgG antibody staining and the FCV N IgM antibody staining generally were similar. FCV G1 IgM antibodies were detected in serum samples from 21 patients (84%). No FCV G1 IgM antibodies were detected in serum samples from 4 patients (16%). In four patients, the FCV G1 IgG and the FCV G1 IgM antibody staining were of similar intensity. In the other 21 patients, the FCV G1 IgM antibody reactivities were considerably less strong than the FCV G1 IgG reactivities.

TABLE 3. Distribution of FCV antibody reactivities among patients and controls

Intensity	No. (%) positive					
	FCV-infected patients (n = 25)				Controls (n = 128)	
	N IgG	N IgM	G1 IgG	G1 IgM	N IgG	G1 IgG
4+	11 (44)	12 (48)	3 (12)	0 (0)	0 (0)	0 (0)
3+	9 (36)	7 (28)	4 (16)	0 (0)	1 (1)	0 (0)
2+	4 (16)	3 (12)	12 (48)	3 (12)	3 (2)	1 (1)
1+	1 (4)	3 (12)	6 (24)	18 (72)	5 (4)	2 (2)
0	0 (0)	0 (0)	0 (0)	4 (16)	121 (93)	125 (98)

Type specificity of FCV N and G1 antibodies. All IgG and IgM antibodies that reacted with the FCV N protein encoded by pFCV-S-1224 also reacted with the PUU N protein encoded by pPUU-N. The intensities of the antibody staining of the FCV N and PUU N proteins were similar (Fig. 1, lanes FCV S1 and PUU S1). pFCV-S-1224 encodes amino acids (aa) 1 through 408 of the FCV N protein, and pPUU-N encodes aa 1 through 433 (the entire polypeptide) of the PUU N protein. Reactivity with the PHV N protein encoded by pPHV-S-1131 generally was much less intense than the reactivities with the FCV and PUU N proteins (Fig. 1C and D, lanes FCV S1 and PHV S1). pPHV-S-1131 encodes aa 41 to 408 of the PHV N protein. To determine whether the PHV N protein contains a cross-reactive epitope in its amino-proximal segment, pPHV-S-330, which expressed PHV N aa 1 to 110, was made. IgG and IgM antibodies that reacted strongly with pFCV-S-1224 and pPUU-N proteins also reacted strongly with pPHV-S-330 (data not shown). These findings suggest that there is a type-common epitope, contained either entirely or in part, within the first 41 aa of the N proteins of FCV, PUU, and PHV that is recognized by FCV antibodies.

Serum IgG and IgM antibodies that reacted with the FCV G1 protein encoded by pFCV-M-1275 (aa 32 to 452) did not react with the PUU G1 protein encoded by pPUU-G1 (aa 1 to 447) or with the PHV G1 protein encoded by pPHV-M-1275 (aa 32 to 452). Therefore, FCV infections generate antibody responses to the FCV G1 glycoprotein that are type specific relative to PUU and PHV (Fig. 1, lanes FCV M1, PHV M1, and PUU M1).

Eight of 25 HPS serum samples were tested for the presence of antibody reactivities to the FCV G1/G2 protein encoded by pFCV-M-2028. One serum sample contained IgG antibodies that reacted with the protein at an intensity level of 3+; no IgM reactivity was detected (data not shown). In the other seven serum samples, no reactivity was observed. Because serum samples from most HPS patients were available in limited quantities, and because G2 antibodies in acute cases of HPS appeared to be uncommon, further studies of FCV G2 antibody responses were deferred.

Localization of the FCV G1 antibody-reactive region. The FCV G1 type-specific epitope was mapped by using nested sets of serially deleted fusion proteins. The amino-terminus boundary was determined by using fusion proteins that contained amino-to-carboxy-terminus deletions. The carboxy-terminus boundary was determined similarly by using fusion proteins that contained carboxy-to-amino-terminus deletions. The nucleotide and amino acid coordinates of the deletion constructs are displayed in Table 2. The reactivities of one FCV G1-reactive serum sample with selected G1 deletion constructs are shown in Fig. 2. FCV G1-reactive antibodies reacted with the pFCV-M-NEx222 protein (aa 59 to 452) and did not react with the pFCV-M-NEx 297 protein (aa 84 to 452) (Fig. 2, lanes 2 and 3, respectively). All constructs that were deleted beyond aa 84 did not react. Therefore, the amino-terminus boundary of the epitope lies between aa 59 and 84. FCV G1 antibodies reacted with the pFCV-M-CEx316 protein (aa 32 to 89) and did not react with the pFCV-M-CEx237 protein (aa 32 to 62) (Fig. 2, lanes 5 and 6, respectively). All constructs that were deleted beyond aa 62 did not react. Therefore, the carboxy-terminus boundary of the epitope lies between aa 62 and 89. The mapping of the amino-terminus boundary and the carboxy-terminus boundary of the type-specific epitope(s) local-

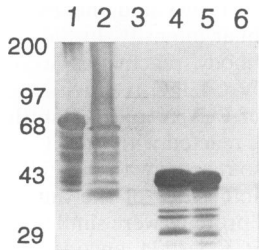

FIG. 2. FCV G1 type-specific epitope mapping. An FCV G1 IgG antibody reactivity was mapped by using deleted fusion proteins. Lanes: 1, FCV G1 pFCV-M-1275 protein; 2 and 3, FCV G1 amino-to-carboxy-terminus deletion constructs pFCV-M-NEx222 and pFCV-M-NEx297, respectively; 4 through 6, FCV G1 carboxy-to-amino-terminus deletion constructs pFCV-M-CEx372, pFCV-M-CEx316, and pFCV-M-CEx237, respectively. See Table 2 for a detailed description of the expression constructs. Numbers at the left mark the mobilities of protein molecular mass standards in kilodaltons.

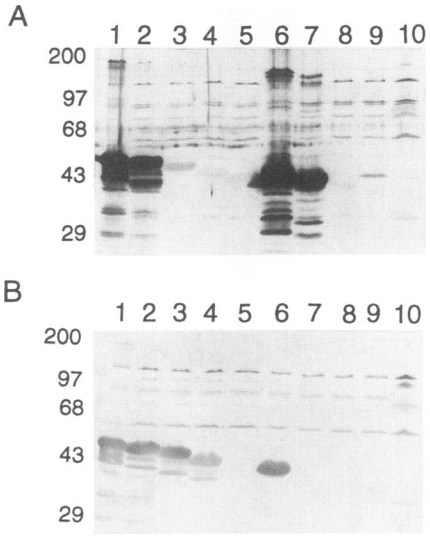

FIG. 3. FCV N IgG epitope mapping among patients and control subjects. The Western blots were reacted with an HPS patient serum (A) and with a control serum (B). Lanes: 1, FCV N pFCV-S-330 protein; 2 to 5, amino-to-carboxy-terminus deletion constructs pFCV-S-NEx91, pFCV-S-NEx136, pFCV-S-NEx181, and pFCV-S-NEx220, respectively; 6 to 9, carboxy-to-amino-terminus deletion constructs pFCV-S-CEx246, pFCV-S-CEx220, pFCV-S-CEx170, and pFCV-S-CEx125, respectively; 10, lysate of bacteria that contain the expression vector pATH10. See Table 2 for a detailed description of the expression constructs. Numbers at the left mark the mobilities of protein molecular mass standards in kilodaltons.

izes the reactivity to a single segment between aa 59 and 89 (the amino acid sequence coordinates are given in terms of the homologous positions in the sequence of PHV). The FCV G1 amino acid sequence of this segment is LKIESSCNFDLH VPATTTQKYNQVDWTKKSS. The sequence is divergent from the homologous regions of PHV (LKLESSCNFDVHTS SATQQAVTKWTWEKKAD) and PUU (LKLESSCNFDLH TSTAGQQSFTKWTWEIKGD).

Localization of FCV N antibody-reactive regions. In addition to the FCV N construct pFCV-S-1224 (aa 1 to 408), three smaller FCV N fusion proteins were tested. These constructs were named pFCV-S-330 (aa 1 to 110), pFCV-S-1131 (aa 41 to 408), and pFCV-S-752 (aa 157 to 408). Both IgG and IgM antibody reactivities to pFCV-S-330 and pFCV-S-752 were detected in HPS serum samples (Fig. 1, lanes FCV S3 and S4, respectively). These constructs represent amino-proximal and carboxy-proximal regions of the FCV N protein, respectively, and their polypeptide segments do not overlap. Therefore, human antibodies induced by FCV infection recognize more than one N protein epitope. Among these three smaller constructs, antibody reactivities to the pFCV-S-330 protein were the strongest.

A segment of pFCV-S-330 recognized by antibodies from five HPS subjects was mapped by using deleted FCV fusion proteins (Table 2). For all five subjects, FCV N IgG antibodies reacted with the pFCV-S-CEx220 protein (aa 1 to 59) and did not react with pFCV-S-CEx170 protein (aa 1 to 41) (Fig. 3A, lanes 7 and 8, respectively). These reactivities placed the carboxy-terminus boundary of the epitope between aa 41 and 59. For two of five subjects, FCV N IgG antibodies reacted with the pFCV-S-NEx91 protein (aa 17 to 110) and did not react with pFCV-S-CEx136 protein (aa 32 to 110) (Fig. 3A, lanes 2 and 3, respectively). These reactivities placed the amino-terminus boundary of the epitope between aa 17 and 32. For these two subjects, the mapping data localized an epitope between FCV N aa 17 and 59. The amino acid sequence of this segment is QLVTARQKLKDAERAVELDPDDVNKSTLQS RRAAVSALETKLG. For the other three HPS subjects, antibody reactivity was observed to all of the amino-to-carboxy-terminal deletions that were tested (extending to aa 60). This finding suggests that the N segment in pFCV-S-330 contains a second epitope that is located closer to the carboxy terminus relative to the epitope between aa 17 and 59. Data presented above suggest that the N epitope located between aa 17 and 59 may be a dominant epitope that is responsible for the cross-reactivity of FCV N antibodies with PHV and PUU N proteins. Confirmation of this hypothesis will require detailed mapping of the FCV N antibody-reactive epitopes in the PHV and PUU N proteins.

Characterization of FCV N IgG and FCV G1 IgG antibody reactivities among control subjects. Nine of 128 (7%) control serum samples contained antibodies that reacted with the FCV N protein encoded by pFCV-S-1224. In four of these subjects, the intensity of the reactivity was greater than or equal to 2+. One of the N-reactive control serum samples also contained IgG antibodies that reacted with the FCV G1 pFCV-M-1275 protein. These four samples were studied for the locations of their N-reactive epitopes. In all four cases, the antibodies recognized pFCV-S-1224 and pFCV-S-330. The FCV N antibody reactivities in the control serum samples did not cross-react with the PUU N protein, in contrast to the FCV N antibodies present in HPS sera. The antibody-reactive epitope was mapped by using deleted FCV fusion proteins. In all four subjects, the N-reactive antibodies reacted with the pFCV-S-CEx246 protein (aa 1 to 68) and did not react with the pFCV-S-220 protein (aa 1 to 59) (Fig. 3B, lanes 6 and 7, respectively). This pattern of reactivity was clearly different from that observed with HPS antibodies, which recognized both the pFCV-S-CEx246 and pFCV-S-CEx220 proteins with similar intensities (Fig. 3A, lanes 6 and 7, respectively). The amino-terminus boundary of the epitope recognized by control serum antibodies mapped between aa 47 and 60 (Fig. 3B, lanes 4 and 5, respectively). These data placed the N-reactive epitope recognized by control serum antibodies between aa 47 and 68. This segment overlaps the N-reactive region (aa 17 to 59) recognized by HPS serum antibodies. More detailed mapping studies are under way to determine whether the minimal epitopes recognized by HPS antibodies and those

recognized by control serum antibodies overlap or are discrete. FCV G1 IgG antibody reactivities present among control serum samples did not react with any of the carboxy-to-amino-terminus deletion constructs that were tested (data not shown). Therefore, the epitope(s) recognized by control serum antibodies lies carboxy proximal to aa 107 and is distinct from the region that contains the FCV G1 type-specific epitope (aa 59 to 89).

DISCUSSION

FCV infections that resulted in HPS induced both N and G1 antibody responses in all cases tested. Strong N and G1 antibody reactivities were detected at initial hospitalization, or at autopsy in cases for which premortem serum samples were not available. Both IgG and IgM antibodies to FCV N were detected in all cases. FCV G1 IgG antibodies were present in all cases, but FCV G1 IgM antibodies were not detected in four cases. These findings are consistent with studies of PUU protein-specific antibody responses in which high levels of N and G1 antibodies were detected during the acute phase of PUU infections (8). FCV N antibodies cross-reacted with PUU and PHV N proteins. FCV G1 antibodies were type specific relative to the G1 proteins of PUU and PHV. These findings suggest that FCV-induced IgM antibodies that react with Hantaan virus, Seoul virus, PUU, and PHV lysates are cross-reactive N antibodies. We conclude that FCV G1 antibodies provides a type-specific diagnosis of FCV infection, whereas antibody reactivities to the FCV N protein do not clearly differentiate FCV infections from infections with other hantaviruses.

FCV G2 IgG antibodies were detected in one of eight HPS serum samples that were tested. HPS serum samples were available in very limited quantities. Because FCV G2 antibody reactivities in acute HPS serum samples appeared to be uncommon, further testing was deferred. Studies of PUU infections suggest that PUU G2 antibodies do not appear until 6 weeks after onset of clinical symptoms (8). Therefore, FCV G2 antibodies may be detected more commonly in HPS serum samples obtained during convalescence.

Serum samples from 128 control subjects were tested for FCV N and G1 IgG antibody reactivities. Only one control sample (0.8%) reacted with both the FCV N and FCV G1 recombinant proteins. This is in marked contrast to the acute HPS cases, in which 100% of the serum samples contained both FCV N and FCV G1 IgG antibodies. Nine of 128 control samples (7%) contained FCV N antibody reactivities. These N antibodies mapped to a site that was different from the epitope recognized by FCV-induced antibodies. Therefore, it seems unlikely that the N-reactive antibodies present among controls resulted from remote unrecognized FCV infections. It is possible that they represent cross-reactive antibodies induced by infection with a different, perhaps uncharacterized, hantavirus. It seems more likely that they represent antibodies induced by an unrelated antigen that fortuitously cross-react with the FCV N protein. The observation that control antibodies and FCV-induced antibodies recognize different FCV N epitopes may provide a means for differentiating false-positive from true-positive FCV N antibody reactivities.

Epitope mapping localized the type-specific FCV G1 antibody response to a single region near the amino terminus of G1 (aa 59 to 89). In HPS, FCV N antibodies that react with more than one segment of the N polypeptide are generated. The strongest N antibody reactivity was mapped to a region near the amino terminus of N (aa 17 to 59). Further studies are under way to map the locations of other FCV N epitopes and to determine whether individual FCV N antigens induce cross-reactive or type-specific antibody responses.

For Hantaan virus and PUU, polyclonal antibody responses and monoclonal antibodies have been studied for type specificity, for the ability to neutralize virus infectivity in vitro, and for the ability to protect experimental animals from virus challenge (1, 2, 7, 13, 15, 16, 20–22, 25–30). In general, N responses have been complex and have included both cross-reactive and type-specific antibodies. N antibodies are not virus neutralizing, and passive transfer of N antibodies does not protect recipient experimental animals from subsequent virus challenge. However, immunization of experimental animals with recombinant N proteins protects the immunized animals from subsequent virus challenge (21, 28). Therefore, the immune response to N protein may include protective cell-mediated immune responses. G1 and G2 responses generally include antibodies that are type specific, that neutralize virus infectivity in vitro, and that protect experimental animals from subsequent infection. Lundkvist et al. found that human antibodies induced by PUU infection compete for antigen binding with neutralizing PUU G1 and G2 monoclonal antibodies, suggesting that the human antibodies recognize a similar epitope (16). The type-specific FCV G1 epitope that we have identified may also mediate protective antibody responses. This information may be useful in future efforts to design FCV vaccines.

The region of the FCV M RNA segment that encodes the G1 type-specific epitope is highly variant relative to other hantaviruses. This region may be the site of frequent viable mutations, or variations within this region may be of selective advantage. As hantavirus isolates from throughout North America are studied more intensively, it will be important to examine the G1 epitope coding segment for changes that could result in antigenic variation.

We have developed recombinant proteins that are useful for the diagnosis of HPS, a severe disease caused by a newly identified hantavirus that we refer to as FCV. Data obtained by studying FCV antibody responses among confirmed HPS cases have been used to develop criteria for the prospective diagnosis of HPS. Serum samples are judged to contain FCV antibodies if (i) both IgG and IgM antibodies that react with the FCV N protein are detected and (ii) IgG antibodies that react with the FCV G1 protein are present. These reagents should be useful tools also in epidemiologic studies of FCV infection prevalence. It is likely that human FCV infections are uncommon, even within populations from which HPS cases have been identified. Therefore, the positive predictive value of an FCV antibody test will depend largely upon the specificity of the assay. Among HPS cases, FCV N antibodies are present in high titer but cross-react with closely related hantaviruses. We also detected FCV N antibody reactivities among 7% of control subjects. An FCV N antibody test alone would have provided a sensitive assay for FCV infection, but the specificity would not have been sufficient for providing accurate estimates of FCV disease prevalence. Among HPS cases, FCV G1 antibodies are present in lower titers but are type specific. The FCV G1 antibody prevalence among control subjects was 3%. For estimating the prevalence of FCV infections, we propose the following strategy. Human serum antibodies will be reacted with FCV N proteins in a rapid screening format. Reactive serum samples will be tested for FCV N and FCV G1 antibodies in Western immunoblot assays. FCV antibody reactivities will be mapped to determine whether the antibodies recognize epitopes that are characteristic of FCV infection. This strategy may also be useful in providing accurate estimates of FCV infection prevalence among rodent populations.

It is hoped that information about FCV infection prevalence among rodents may increase our understanding of the circumstances that result in transmission to humans.

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