JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 600–608 Vol. 35, No. 3
0095-1137/97/$04.00+0

Rapid and Specific Detection of Sin Nombre Virus Antibodies in Patients with Hantavirus Pulmonary Syndrome by a Strip Immunoblot Assay Suitable for Field Diagnosis

BRIAN HJELLE,[1,2]* STEVEN JENISON,[2,3]† NORAH TORREZ-MARTINEZ,[1] BRUCE HERRING,[4] STELLA QUAN,[4] ALAN POLITO,[4] SERGIO PICHUANTES,[4] TAKASHI YAMADA,[3]‡ CAROL MORRIS,[3]§ FREDRIK ELGH,[5] HO WANG LEE,[6] HARVEY ARTSOB,[7] AND ROBERT DINELLO[4]

Departments of Pathology[1] and Medicine,[3] Hantavirus Diagnostic Unit, and Cancer Research and Treatment Center,[2] University of New Mexico School of Medicine, Albuquerque, New Mexico; Chiron Diagnostics, Emeryville, California[4]; Department of Virology, University of Umeå, Umeå, Sweden[5]; Asan Institute for Life Sciences, Seoul, Korea[6]; and Laboratory Centre for Disease Control, Ottawa, Ontario, Canada[7]

Received 11 June 1996/Returned for modification 20 August 1996/Accepted 9 December 1996

To develop a rapid antibody test for Sin Nombre hantavirus (SNV) infection for diagnosis of hantavirus pulmonary syndrome (HPS) in field settings where advanced instrumentation is not available, a strip immunoblot assay bearing four immobilized antigens of SNV and a recombinant nucleocapsid protein antigen of Seoul hantavirus (SEOV) was prepared. The SNV antigens included a full-length recombinant-expressed nucleocapsid (N) protein (rN), a recombinant-expressed G1 protein (residues 35 to 117), and synthetic peptides derived from N (residues 17 to 59) and G1 (residues 58 to 88). On the basis of the observed reactivities of hantavirus-infected patient and control sera, we determined that a positive assay requires reactivity with SNV or SEOV rN antigen and at least one other antigen. Isolated reactivity to either viral rN antigen is indeterminate, and any pattern of reactivity that does not include reactivity to an rN antigen is considered indeterminate but is unlikely to represent hantavirus infection. Fifty-eight of 59 samples from patients with acute SNV-associated HPS were positive according to these criteria, and one was initially indeterminate. Four of four samples from patients with HPS due to other hantaviruses were positive, as were most samples from patients with SEOV and Puumala virus infections. Of 192 control serum samples, 2 (1%) were positive and 2 were indeterminate. Acute SNV infection was distinguishable from remote SNV infection or infection with hantaviruses other than SNV by the presence of G1 peptide antigen reactivities in the former. The strip immunoblot assay shows promise for the detection of SNV antibodies early in the course of HPS.

Hantaviruses (*Bunyaviridae*: *Hantavirus*) are enveloped, minus-sense RNA viruses with a tripartite genome. The large, middle, and small genomes encode an RNA-dependent RNA polymerase, envelope glycoproteins G1 and G2, and a core (nucleocapsid [N]) protein, respectively. Nearly 20 distinct hantaviruses are found in association with specific rodent or insectivore hosts worldwide. Their modes of transmission to humans, their natural reservoirs, and the clinical features of human infection have recently been reviewed (32).

Hantavirus pulmonary syndrome (HPS) was first recognized in association with a series of unexplained deaths among rural residents of the Four Corners region of the United States, where the states of New Mexico, Arizona, Utah, and Colorado meet. The disorder was remarkable for its rapid progression from mild febrile prodrome to noncardiogenic pulmonary edema, shock, and death (8). A heretofore unknown viral species, Sin Nombre virus (SNV; also known as Four Corners virus and Muerto Canyon virus) was shown to be the etiologic agent through both serologic and PCR investigations (36). Deer mice, especially *Peromyscus maniculatus*, were identified as the predominant carrier of SNV (6, 35). The virus was ultimately isolated from deer mice from New Mexico and California (10, 41).

Although three other North American hantaviruses have been associated with HPS, New York virus (NYV), Black Creek Canal virus (BCCV), and Bayou virus (BAYV), it is believed that infection with SNV and closely related viruses may account for as many as 96% of the 165 cases recorded of HPS in North America as of October 1996 (4, 21, 24, 25, 34, 40, 43). Cases of HPS have been reported in 25 U.S. states and three Canadian provinces (5, 44, 46). Worldwide, a larger toll of illness is caused by the Eurasian hantaviruses that cause hemorrhagic fever with renal syndrome (HFRS). HFRS-associated viruses include Hantaan virus (HTNV), Puumala virus (PUUV), Seoul virus (SEOV), and Dobrava-Belgrade virus (DOBV) (1, 2, 26, 27). Of these, only SEOV, which is carried by rats that live commensally with humans (*Rattus* spp.), occurs in the Western Hemisphere and has caused human disease in the United States (12, 13).

A variety of formats for serologic diagnosis of hantavirus infection have been used, including high-density particle agglutination, immunofluorescence and immunoprecipitation assays with laboratory-cultivated viruses, hemagglutination inhibition assay, plaque- and focus-reduction neutralization assays, Western blotting (immunoblotting), and enzyme-linked immunosorbent assay (ELISA) (7, 9, 17, 23, 26, 30, 31, 42, 45). Recently, there has been an increased use of recombinant-expressed viral proteins as antigenic targets. In recombinant

* Corresponding author. Mailing address: Department of Pathology, University of New Mexico School of Medicine, Albuquerque, NM 87131-5301. Phone: (505) 277-0624. Fax: (505) 277-1950. E-mail: Brian_Hjelle@somasf.unm.edu.

† Present address: HIV/AIDS/STD Bureau, Santa Fe, NM 87501.

‡ Present address: 4-13 Nishi-17-jo-Kita-3-chome, Obihiro City, Hokkaido 808, Japan.

§ Present address: Department of Microbiology, University of New Mexico, Albuquerque, NM 87131.

TABLE 1. Characteristics of serum samples from patients with hantavirus infection examined in the present study

Category	Type of sample[a]	Location of occurrence	Time after onset of lung disease of sample draw	PCR-verified viral diagnosis[b]
SNV-associated HPS and presumed SNV-associated HPS	Acute phase (59)[c]	Canada (7), United States (52)[d]	−1 (3), 0 (19), 1 (10), 2 (11), 3 (5), 5 (2), <6 (1), 7 (4), 8 (1), and <11 (3) days	SNV (36), negative (1), untested (22)
Presumed SNV-associated HPS[e]	Convalescent phase or remote infection (16)	Canada (2), United States (14)	14 (1), 16 (1), and 22 (1) days; 1–12 mo (7); and 2 (2), 5 (1), 10 (1), 19 (1), and 36 (1) yr	Untested (16)
NYV-associated HPS	Acute phase	New York (1)	1 days (1)	NYV (1)
BAYV-associated HPS	Acute phase	Texas (2)	4 (1) and 5 (1) days	BAYV (2)
BCCV-associated HPS	Acute phase	Florida (1)	7 days (1)	Untested (1)
HTNV-associated HFRS	Acute phase	Korea (7)	7–14 days (7)	Untested (7)
SEOV-associated HFRS	Acute phase	Japan (17)	7–14 days (17)	Untested (17)
PUUV-associated HFRS	Acute phase	Sweden (10)	7–24 days (10)	Untested (10)
PUUV-associated HFRS	Acute phase	Finland (6)	7–21 days (6)	Untested (6)

[a] An acute-phase serum sample is defined as one drawn within 11 days of the onset of lung symptoms or abnormalities on chest X ray.
[b] PCR amplification of viral genome with sequence confirmation of virus species was conducted with peripheral blood mononuclear cells, serum, a blood clot specimen, or lung tissue-derived RNA template.
[c] Numbers in parentheses represent the number of serum specimens.
[d] Of the acute-phase specimens from Canada, four were from Alberta and three were from British Columbia. Of the acute-phase specimens from the United States, 15 were from Arizona, 6 were from California, 1 was from Colorado, 3 were from Idaho, 2 were from Kansas, 1 was from Montana, 14 were from New Mexico, 2 were from Nevada, 1 was from Oregon, 3 were from Texas, 1 was from Utah, and 2 were from Washington.
[e] No acute-phase serum specimens were available from these patients. Both convalescent-phase specimens from Canada were from Alberta. Of the convalescent-phase specimens from the United States, four were from Arizona, one was from California, three were from New Mexico, two were from South Dakota, one was from Texas, one was from Utah, one was from Washington, and one was from Wyoming.

antigen diagnostic assays, the viral N antigen is dominant over the viral glycoproteins. Antibodies to the N antigen arise early in the course of infection and are universally detectable in convalescence (9, 14, 23, 30, 31, 49, 50). Antibodies to glycoprotein antigens are traditionally detected in neutralization assays, in hemagglutination inhibition assays, and possibly in immunofluorescence assays. Human infection with SNV is universally associated with antibodies to the viral G1 antigen, as detected by Western blotting. SNV G1 antibodies are not reactive with the G1 antigens of other hantaviruses (17, 23).

Among the pathogenic hantaviruses, HTNV, SEOV, and DOBV are antigenically similar. The HPS-associated viruses are also closely related to one another and cross-react best with PUUV. Antigenic cross-reactivity is most pronounced among the viral N proteins.

Advances in tertiary care management that may reduce the mortality of HPS have occurred (32). Since HPS progresses very rapidly, these advances are likely to affect the prognosis only for those patients for whom the diagnosis can be made in a timely manner. A method for the rapid detection of SNV antibodies appropriate for a rural setting and that could be applied during the early stages of illness could improve the prospects for early intervention. We considered that the ideal test would be simple and would be one that does not require specialized equipment such as ELISA readers or expertise such as that required for immunofluorescence assays.

MATERIALS AND METHODS

Patients and controls. Serum samples from patients with HPS were selected on the basis of their availability in sufficient quantity to our laboratory. All HPS patient samples came from patients who met the U.S. Public Health Service's case definition for HPS (4) and are recognized as HPS patients by the U.S. Centers for Disease Control and Prevention (CDC) or the Canadian Laboratory Centre for Disease Control. Most HPS samples were submitted to the University of New Mexico Hantavirus Diagnostic Unit for routine diagnosis. Others were solicited from outside laboratories after the diagnosis was made elsewhere, usually by ELISAs. HPS patient samples are listed in Table 1 according to sample type (acute-phase infection or convalescent-remote infection), the etiologic viral serotype, and results of PCR investigations. For our purposes, an acute-phase specimen is one collected within 11 days of the onset of lung disease.

Hantaviruses other than those associated with HPS were also examined. Serum samples were obtained from 17 Japanese patients with SEOV-induced HFRS, 6 Korean patients with HTNV-associated HFRS, and 10 Swedish and 6 Finnish patients with PUUV-associated HFRS (nephropathia epidemica). Swedish HFRS serum samples were collected between 7 and 24 days from the first day of hospitalization. Although the precise timing between illness and serum collection is not known for the Finnish and Japanese HFRS patient samples, these were all collected between 7 and 21 days after hospitalization (Table 1).

Swedish patients with PUUV-associated HFRS were diagnosed on the basis of PUUV recombinant N (rN) ELISA and PUUV immunofluorescence assay (9). HTNV- and SEOV-associated HFRS and Finnish PUUV-associated HFRS patients were diagnosed by immunofluorescence assays and ELISAs, and the etiologic agents were distinguished on the basis of the clinical setting and serologically by their reactivities in focus reduction neutralization tests and Western blots (28, 29, 45a).

Control samples consisted of 192 sequential samples that were originally collected in 1991 and that were submitted to an Indian Health Service hospital laboratory for various chemical or immunological studies. These samples comprised a subset of 446 sequential samples (group 2 in the report of Hjelle et al. [15]).

RIBA SIA. The hantavirus strip immunoblot assay (SIA; Chiron Corporation) incorporates four recombinant-expressed and synthetic peptide antigens of SNV, as well as a recombinant-expressed N antigen of SEOV. The membrane consisted of nitrocellulose with an inert plastic backing as a support. Antigens were applied to the membrane in lanes, adjacent to two lanes containing two levels of human immunoglobulin G (IgG) (level I, low control, and level II, high control) as internal controls (Fig. 1).

The strips were incubated at room temperature for 4 to 4.5 h in a 1:50 dilution of human serum in a specimen diluent buffer (phosphate-buffered saline [PBS; pH 7.4] with detergents and bovine serum albumin [BSA]) essentially as described previously (23). After one washing in specimen diluent, followed by three washings in a PBS detergent wash solution (pH 7.4) (23), the strips were exposed to a secondary horseradish peroxidase-conjugated goat anti-human heavy and light chain antibody for 9 to 11 min at room temperature, followed by three more washings and exposure to a horseradish peroxidase substrate (4 chloro-1-naphthol), as recommended by the manufacturer. After 15 min, the substrate was decanted and the strips were rinsed twice in deionized water.

A serum sample was considered reactive against a given antigen only if reactivity was greater than or equal to that of the level I IgG control band, which is defined to represent a 1+ reactivity. A reactivity equivalent to that of the level II IgG control band was considered to represent a reactivity of 3+. Reactivity intensity intermediate between those of the level I and level II IgG control bands is considered to be 2+, and reactivity stronger than that of the level II band is considered to be 4+. The control bands provide a consistent semiquantitative standard against which the reactivities to hantavirus antigens can be measured and allow for a firm definition for positive band reactivity.

The preparation of the antigens was as described below.

(i) IgG controls. The level I control consisted of 16 ng of human IgG and level II consisted of 80 ng of human IgG for each SIA strip.

(ii) SNV rN. The immunodominant and cross-reactive SNV N antigen (genotype 3H226) is incorporated as a full-length recombinant protein in fusion with

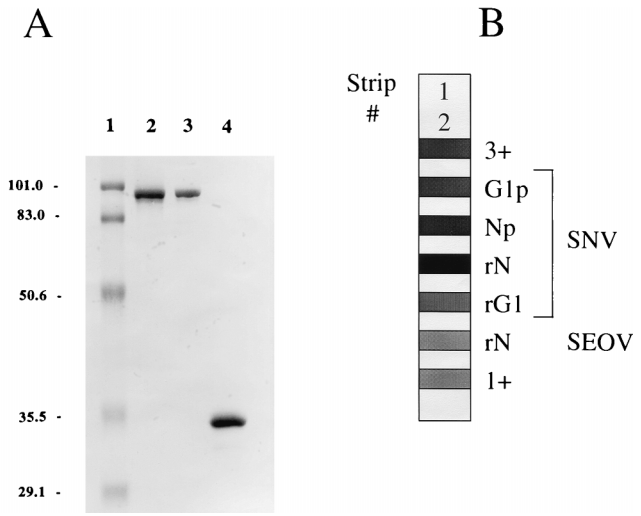

FIG. 1. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified recombinant antigens used in the SIA. A 14% gel was loaded with molecular weight markers (Bio-Rad; lane 1), ~0.5 μg of SEOV rN (lane 2), ~0.3 μg of SNV rN (lane 3), and ~0.5 μg of SNV rG1 (lane 4) and was stained with Coomassie blue. (B) Identities and locations of the antigens coated on the strip. Two levels of human IgG are included in each strip as internal controls. The reactivity of each antigen bands was determined by comparing the intensity of each band to the level I (1+) and level II (3+) internal controls in each strip as described in the text. Reactivities approximating those obtained with the positive control serum sample supplied with the kit are shown. G1p and Np, G1 and N peptide antigens, respectively; rN and rG1, recombinant N and G1 antigens, respectively; the viruses from which each antigen is derived (SNV 3H226 or SEOV 80/39) are indicated.

the 50-kDa *Escherichia coli* maltose binding protein (MBP) in the vector pMal C2 (Novagen, Madison, Wis.). The 100-kDa MBP-SNV N antigen was affinity purified by column chromatography on amylose resin and elution with maltose, according to the manufacturer's instructions (48). As with all of the recombinant antigens, purity was judged visually after overloading on a sodium dodecyl sulfate-polyacrylamide gel and staining the gel with Coomassie brilliant blue (Fig. 1A). Ninety nanograms of rN protein was used in each strip.

(iii) SNV rG1. Since expression of extensive portions of the 652-amino-acid (aa) G1 was hampered by instability and variable expression, a smaller (82-aa) portion of SNV G1 (residues 35 to 117 relative to the position of the initiating methionine of genotype 3H226) was used in fusion with the 153-aa yeast superoxide dismutase (SOD). The small G1 fragment was synthesized by PCR from pFCV-M-1275 (48) by using primers that introduced an *Nco*I site on the 5′ end and a *Sal*I site and two stop codons on the 3′ end. The 268-nucleotide (nt) *Nco*I-*Sal*I-digested fragment was used to replace the 347-nt insert of pSOD-HIV2PR113 (38), creating the construct pSOD-G1. A 603-nt *Stu*I-*Sal*I fragment of pSOD-G1 was ligated into the vector pSI1-PR179 (37) to provide the ADH2-GAPDH promoter. The 2,093-nt *Bam*HI-*Sal*I cassette of the resulting construct was then introduced into the yeast expression vector pBS24.1 to create the final expression construct pSOD-SNV-G1. This was expressed as a 23-kDa SOD-G1 fusion in the protease-deficient strain *Saccharomyces cerevisiae* AB122 (*MAT* **a** *leu2 ura3-52 prb1-1122 pep4-3 prc1-407* [cir^0]) (39). The 82-aa portion of G1 expressed in this construct spans the immunodominant epitope (residues 58 to 88 [23]) and contains some additional flanking sequence from G1.

The yeast cells were disrupted in PBS–1 mM EDTA (pH 7.2) with a Dyno-Mill and glass beads (Glen Mills Inc.), and the highly insoluble recombinant G1 (rG1) was recovered by extensive washing with the same buffer containing 4 M urea. The protein was dissolved in PBS–EDTA at pH 11.5. After neutralization the protein was concentrated and further purified by ammonium sulfate precipitation, solubilized, and subjected to gel filtration chromatography on a Sephacryl S-200 resin (Phamacia, Piscataway, N.J.). One hundred eighty nanograms of recombinant protein was added to each strip.

(iv) SEOV rN. A 1,272-nt segment of the SEOV (strain 80-39) N gene encoding residues 1 through 424 of the 429-aa protein was inserted in pMal C2 and was expressed in fusion with MBP. The 100-kDa fusion protein was purified by chromatography over an amylose column. Ninety nanograms of recombinant protein was loaded per strip.

(v) SNV G1 peptide. A 31-aa peptide encoded by the 3H226 allele of SNV G1 was synthesized by 9-fluorenymethoxycarbonyl chemistry and was conjugated to BSA. For conjugation, 3 mg of peptide was suspended in 0.95 ml of PBS (pH 7.4)–2 mM EDTA–6 M guanidinium chloride and was reduced by the addition of 50 μl of 1 M dithiothreitol for 1 h at room temperature. After purification on a G10 Sephadex column, the peptide was conjugated with BSA-maleimide at a ratio of 10:1 (mole/mole). Unreacted maleimidyl groups were blocked with 10 mM 2-mercaptoethylamine at room temperature for 1 h. The solution was diluted with 9 volumes of PBS containing 2 mM EDTA and 2 M guanidinium chloride and was then concentrated with a 10-ml Amicon cell and YM30 filter to a volume of 1 ml. The concentration step was repeated after redilution with another 9 volumes of the same solution. The protein concentration was determined, and it was loaded at 90 ng/strip.

The G1 peptide sequence was chosen on the basis of epitope mapping studies that showed that residues 58 to 88 of SNV constitute an immunoreactive linear domain that is recognized by SNV antibodies, rarely by SEOV antibodies, and not by antibodies to PUUV or HTNV (21a, 23, 48).

(vi) SNV N peptide. A 43-aa peptide of the 3H226 N protein was synthesized on the basis of previous studies that showed that residues 17 to 59 of SNV contain the large majority of immunoreactivity directed against the SNV N antigen, as assessed by Western blot analysis (23). The same protocol used for the G1 peptide was used for conjugation. Ninety nanograms of conjugate was used per strip.

Loading of antigens onto the strip. Proteins and peptide conjugates were diluted in PBS (pH 7.4) and naphthol blue black was added to an optical density at 616 nm of 0.02 to 0.05, and the strip was manually coated. The antigen-PBS solution was placed in the upper well of a custom-built slot blot manifold that is similar to the Minifold II Slot-Blot manifold of Schleicher & Schuell (Keene, N.H.). The solution was drawn through the nitrocellulose membrane with a vacuum of 0.4 atmospheres. After the solution had passed through the membrane, the membrane was removed from the manifold and dried for 24 h at 45°C. The membrane was briefly immersed in 1% casein in PBS and was redried under the same conditions and then applied to a plastic adhesive backing, followed by an additional drying cycle.

Preadsorption with *E. coli* lysate. Serum samples (1:50 in specimen dilution buffer) from patients from the control populations that demonstrated reactivity to any hantavirus antigen in the SIA were subjected to preadsorption for 1 h at room temperature with a 1:40 dilution of *E. coli* lysate, which was prepared as described by Jenison et al. (23). Lysates were prepared from cultures of JM109 cells that were transformed with pMal C2 and after induction of MBP, as described previously (48). The preadsorbed serum samples were then used to probe hantavirus SIA membranes by the standard protocol.

Western blot assays. Western blot assays were performed with selected samples as described previously (23, 48), except that a T7 gene 10–viral fusion protein instead of an MBP fusion protein was used for SNV N. The G1 protein was pFCV-M-1275 (48). The N protein expression construct was prepared by amplifying cDNA from lung RNA (human genotype 3H226) with the primers 5′-**AAGCTT**GGAATGAGCACCCTCAAAGA (sense; position 34 of the small segment) and 5′-**GCTCGAG**ATAAAGTTTAAGTGGTTCTTGGTT (antisense; position 1326). A reverse transcription (RT) step (1 h) was followed by amplification for 35 cycles at 94°C for 1 min, 48°C for 1 min, and then 72°C for 3 min under previously described conditions (16). The N cDNA was cloned at the *Hin*dIII and *Xho*I sites (boldface residues in the primer sequences) into the expression construct pET23b (Novagen). Expression of rN was induced in BL21 (DE3) cells with isopropyl-β-D-thiogalactopyranoside, and rN was affinity purified over a metal-chelating column, as recommended by Novagen. Full-length SNV rN protein was run in one lane, alongside partial rG1 proteins spanning residues 32 to 452 and control bacterial lysate lanes (23, 48). Both IgG and IgM secondary antibodies were used in separate experiments.

RT-PCR. RT-PCR amplification with sequence confirmation was conducted whenever anticoagulated blood specimens, blood clot specimens, or necropsy tissue specimens (lung, heart, or kidney) were collected from a patient during the acute phase of illness. For living patients, anticoagulated blood or blood clot specimens were the preferred source of RNA, which was prepared as described previously (18). In most cases, SNV-specific primers in the G1 gene were used to confirm SNV infection, followed by sequencing of the amplified DNA (17), but for some 1993 case patient samples, G2 primers were used (18, 36). For amplification of hantavirus genomes other than SNV (NYV and BAYV), various generic hantavirus small-segment primers were used when SNV-specific primers failed to produce a PCR product, as previously described (19, 21).

RESULTS

Early and specific detection of SNV-reactive antibodies in the serum of patients with acute HPS. Fifty-nine serum samples from patients with acute SNV-HPS (defined as those collected less than 11 days from the time of development of the patient's first pulmonary symptoms) were available in sufficient quantity (total volume, ≥20 μl) for testing (Table 1). For each patient, the earliest available serum sample was used. Forty-eight (81%) of the 59 samples were collected within the first 3 days of the development of the first pulmonary symptoms, which for nearly all patients was the same as the first day of hospitalization. For several patients the precise date of blood

TABLE 2. Reactivities in hantavirus SIA with serum samples from patients with acute-phase or remote- or convalescent-phase SNV-associated HPS

Serum sample source and frequency[a]	Interval	SIA reactivity				
		G1 peptide	N peptide	N recombinant	G1 recombinant	SEOV N antigen
Acute-phase SNV-associated HPS						
1	−1 d[b]	≥1+	0	≥1+	0	0
2	−1 d	≥1+	≥1+	≥1+	≥1+	0
1	0 d	0	0	≥1+	0	0
1	0 d	≥1+	0	≥1+	0	0
2	0 d	≥1+	0	≥1+	≥1+	0
10	0 d	≥1+	≥1+	≥1+	≥1+	0
3	0 d	≥1+	≥1+	≥1+	0	0
1	0 d	≥1+	0	≥1+	≥1+	0
1	0 d	≥1+	0	≥1+	≥1+	≥1+
1	1 d	≥1+	≥1+	≥1+	0	0
9	1 d	≥1+	≥1+	≥1+	≥1+	0
8	2 d	≥1+	≥1+	≥1+	≥1+	0
3	2 d	≥1+	≥1+	≥1+	≥1+	≥1+
4	3 d	≥1+	≥1+	≥1+	≥1+	0
1	3 d	≥1+	≥1+	≥1+	≥1+	≥1+
2	5 d	≥1+	≥1+	≥1+	≥1+	0
1	<6 d	≥1+	≥1+	≥1+	≥1+	≥1+
4	7 d	≥1+	≥1+	≥1+	≥1+	0
1	8 d	≥1+	≥1+	≥1+	≥1+	≥1+
3	<11 d	≥1+	≥1+	≥1+	≥1+	0
Remote SNV-asso HPS						
1	14 d	≥1+	≥1+	≥1+	≥1+	0
1	16 d	≥1+	≥1+	≥1+	≥1+	0
1	22 d	≥1+	≥1+	≥1+	≥1+	0
2	1 mo	≥1+	≥1+	≥1+	≥1+	0
1	2 mo	0	≥1+	≥1+	0	0
2	3 mo	≥1+	≥1+	≥1+	≥1+	0
1	8 mo	≥1+	≥1+	≥1+	≥1+	≥1+
1	11 mo	0	≥1+	≥1+	0	≥1+
2	2 yr	0	≥1+	≥1+	0	0
1	5 yr	0	≥1+	≥1+	0	0
1	10 yr	0	≥1+	≥1+	0	0
1	19 yr	0	≥1+	≥1+	0	0
1	36 yr	0	≥1+	≥1+	0	0

[a] Number of occurrences of a particular reactivity pattern.
[b] d, days.

sampling relative to the onset of pulmonary disease could not be ascertained, so a conservative estimate of the maximal interval was made on the basis of the length of hospital stay and the time of onset of the first symptoms.

The reactivities to each of the various membrane-bound antigens is indicated in Table 2. The most common pattern, seen in 87% of the samples, showed reactivity of 2+ to 4+ against the SNV rN and N peptide antigens, coupled with reactivity of 1+ to 4+ against the SNV rG1 and/or G1 peptide antigens (data not shown). The stronger reactivities to the N peptide and the G1 antigens were more common in samples collected 1 or more days after the development of pulmonary symptoms. Cross-reactivity to SEOV rN was generally absent or weak.

Another selection of samples came from patients with convalescent-phase or remote SNV-associated HPS. These samples were considered to be related to SNV infections on the basis of (i) appropriate clinical history, including an acute febrile illness featuring myalgia, thrombocytopenia, neutrophilia with left shift, and pulmonary disease with diffuse interstitial involvement on chest X ray; (ii) occurrence of infection in a patient within the habitation range of the deer mouse *P. maniculatus*; and (iii) positive detection of serum IgG antibodies to SNV nucleocapsid by Western blot assays in studies conducted retrospectively (17, 23, 47). RT-PCR studies and/or IgM studies either were negative or were not conducted for these patient samples. Sixteen such samples from patients from whom an acute-phase serum sample was not available were available to us. The interval between the onset of pulmonary disease and collection of the serum ranged from 14 days to 36 years.

A series of 192 control serum samples was obtained from a collection that had been part of a previous retrovirus seroprevalence study (15). These samples were collected in 1991 from an Indian Health Service hospital laboratory in western New Mexico, near the site of the 1993 HPS outbreak. The large majority (97%) of these samples came from patients >20 years of age, and specimens from both inpatients and outpatients were included in the collection. The reactivities of these specimens are listed in Table 3. Reactivity to one or more bands was observed in eight (4.2%) of these specimens.

Virtually all of the acute- or convalescent-stage sera from patients with SNV-associated HPS were reactive against one or both synthetic peptide antigens as well as with the SNV rN antigen (Table 2). In contrast, this pattern was rare for sera from the control population (Table 3). Only two serum samples from that group displayed reactivity to both the SNV rN and SNV N peptide antigens, and neither had reactivity to the viral G1 antigen in either the peptide or the recombinant form. To determine whether some of the reactivities observed in the sera from the control population were due to trace amounts of contaminating bacterial antigens in the rN or SEOV rN bands, all of the eight control serum samples that reacted to any viral band in the SIA were subjected to preadsorption with an *E. coli* lysate containing induced MBP. As expected, only the reactivities of the two samples that reacted exclusively to the MBP-based rN antigen were affected by the pretreatment (data not shown). The reactivity of one sample that had reacted with 2+ intensity before adsorption was reduced to 1+. Another sample that had reacted at 1+ intensity lost all reactivity with preadsorption. Since sera with reactivities to isolated bands other than rN or SEOV rN are considered unlikely to be responsive to hantavirus infection, we did not attempt to identify the determinants responsible for such reactivities.

Additional Western blot studies were conducted with all of serum samples from the control populations that demonstrated reactivity with any antigen band by the SIA. None of the six control samples with single-antigen reactivity on the SIA demonstrated IgG antibodies reactive with SNV N or G1

TABLE 3. Hantavirus SIA reactivities for 192 unselected serum samples from patients at an Indian Health Service facility in western New Mexico

No. of samples[a]	SIA reactivity				
	SNV G1 peptide	SNV N peptide	SNV rN	SNV rG1	SEOV rN
184	0	0	0	0	0
1	≥1+	0	0	0	0
2	0	0	≥1+	0	0
3	0	0	0	≥1+	0
2	0	≥1+	≥1+	0	0

[a] Number of samples with a particular reactivity pattern.

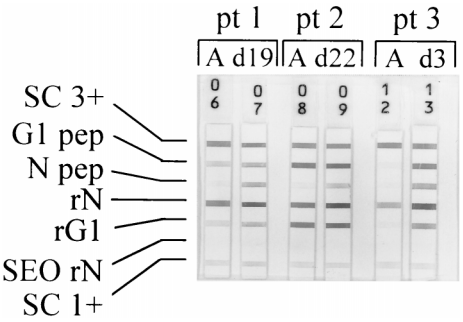

FIG. 2. Temporal evolution of serologic responses in three patients with acute hantavirus pulmonary syndrome due to RT-PCR-confirmed SNV infection. For each patient, an SIA was performed with an admission serum sample (lanes A) and a follow-up sample drawn on day 19 (patient 1), day 22 (patient 2), or day 3 (patient 3). The control bands with high (SC 3+) and low (SC 1+) amounts of IgG are defined to represent 3+ and 1+ levels of reactivity, respectively. Five viral antigen bands are placed internally to the serum control (SC) bands. These include the peptide (pep) and recombinant SNV G1 and N antigens, as well as SEOV rN.

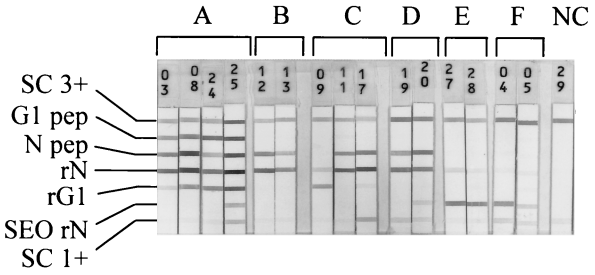

FIG. 3. Representative RIBA SIA strips probed with serum samples from patients acutely infected with different hantaviruses. Group A strips were probed with sera from patients with acute SNV-associated HPS, including a sample from British Columbia (strip 03), a sample from New Mexico (strip 08), a sample from Colorado drawn a day before the onset of respiratory symptoms (strip 24), and a sample from Arizona (strip 25). Group B strips were probed with serum samples obtained in 1995 from patients with remote HPS presumed to be due to SNV, one of whom was ill in 1985 (strip 12) and one of whom was ill in 1959 (strip 13). Group C strips were probed with sera from patients with acute HPS due to viruses other than SNV: NYV (strip 09), BCCV (strip 11), and BAYV (strip 17). Group D, E, and F strips were probed with sera from patients infected with PUUV, SEOV, or HTNV, respectively. NC, negative control. Antigen bands are as identified in Fig. 1B.

antigens by Western blot assay, but both of the samples with rN plus N peptide reactivities had IgG reactivity to recombinant SNV N protein by Western blot assay (data not shown). None of these samples had detectable IgM reactivity to any SNV N antigen.

Evolution of antibody responses in patients with early SNV infection. To determine whether the hantavirus SIA will be able to efficiently detect SNV antibodies in the earliest stages of infection, we used the earliest serum samples available. Three samples were available from patients tested in the prodrome stage of illness (all at day −1 relative to the time of onset of the first lung symptom), and all were positive, as were all but one of the samples drawn at the onset of lung disease or at later times. The single exceptional sample was an EDTA-anticoagulated whole-blood sample that was drawn on the first day of lung symptoms. This sample was recovered from a waste disposal bag and is of uncertain quality. It showed isolated 2+ reactivity against the SNV rN protein (indeterminate pattern), despite a Western blot study that showed 3+ IgM and 3+ IgG reactivities to the SNV rN antigen and trace IgM and 1+ IgG reactivities to the SNV rG1 antigen (data not shown).

Evolution of antibody response in early HPS. The detection of a single plasma sample from a patient with early HPS with an indeterminate pattern of reactivity led us to examine a selected series of paired patient samples, separated by 3 to 22 days, from patients whose earliest available serum samples showed relatively weak reactivities. The limited number of samples in which a significant enhancement of antibody reactivity could be demonstrated showed modest to marked increases in the reactivities to the SNV G1 peptide, N peptide, rN, and rG1 antigens over time (Fig. 2). The patient whose initial response was restricted to the rN antigen developed a strongly positive response to multiple SNV antigens by day 3 of illness (Fig. 2, patient 3).

Reactivity of sera from patients with non-SNV HPS and HFRS. In general, the reactivities observed when hantavirus SIA strips were probed with sera from patients with non-SNV hantavirus infections were consistent with those observed in previous studies by the Western blot technique (17, 23). Among patients with HPS due to viruses other than SNV, acute-phase serum samples from patients with BAYV ($n = 2$), BCCV ($n = 1$), or NYV ($n = 1$) infection failed to react with the G1 peptide of SNV. The sera from patients with BAYV and BCCV infections reacted exclusively with the relatively conserved N peptide and rN antigens, while the serum sample from the patient with NYV infection reacted with the rN and the rG1 antigens (Fig. 3; Table 4). Since rG1 is somewhat larger than the G1 peptide, this reactivity may be directed against a second linear epitope or conformation-dependent epitope of G1 that is rarely if ever recognized in the sera of patients with SNV infection (14b, 23). One serum sample from a patient with SEOV infection also appears to have recognized a G1 epitope outside of that for the immunodominant peptide.

PUUV, which is the agent of HFRS that bears the closest genetic and antigenic relationship to the HPS-associated viruses, elicited antibodies that generally were strongly reactive against the N peptide and rN antigens, with no reactivity against the G1 peptide or rG1 (Table 4). A single serum

TABLE 4. Antibody reactivities in hantavirus SIA with sera from patients with acute NYV, BCCV, BAYV, SEOV, HTNV, and PUUV infections

Virus (no. of samples)	No. of samples[a]	Reactivity				
		SNV G1 peptide	SNV N peptide	SNV rN	SNV rG1	SEOV rN
NYV (1)	1	0	0	≥1+	≥1+	0
BCCV (1)	1	0	≥1+	≥1+	0	0
BAYV (2)	2	0	≥1+	≥1+	0	0
SEOV (17)	3	0	0	0	0	≥1+
	1	0	0	≥1+	0	≥1+
	1	0	0	0	≥1+	≥1+
	7	0	0	≥1+	0	≥1+
	3	0	≥1+	≥1+	0	≥1+
	2	≥1+	0	≥1+	0	≥1+
HTNV (7)	4	0	0	0	0	≥1+
	3	0	0	≥1+	0	≥1+
PUUV (16)	1	0	0	≥1+	0	0
	13	0	≥1+	≥1+	0	0
	2	0	≥1+	≥1+	0	≥1+

[a] Number of samples with a particular reactivity pattern.

sample from a patient with PUUV infection was indeterminate by the SIA, with reactivity to rN in isolation. SEOV antibodies were reactive against the homologous rN antigen, although cross-reactivities with SNV rN and N peptide were also common. Surprisingly, two serum samples from patients with SEOV infection exhibited modest (1+) reactivity against the G1 peptide antigen (Table 4), which was accompanied by trace (<1+) reactivity against the rG1 antigen (data not shown). An additional serum sample from a patient with SEOV infection showed 2+ reactivity against rG1 but did not react with the G1 peptide. Patterns of reactivity observed in sera from patients with HTNV-associated HFRS were similar to those observed in sera from patients with SEOV-associated HFRS, although they were generally somewhat weaker, and no G1 reactivities were observed.

Patterns of SNV seroreactivity in convalescent- and acute-phase serum samples. Although band reactivities as high as 3+ to 4+ in intensity persisted against the SNV N peptide and rN antigens for as long as 36 years, reactivities to the G1 peptide and rG1 antigens appeared to be absent in samples older than ~8 months (Fig. 3; Table 2). Since it could not be unequivocally determined that the etiologic agent in these remote cases was SNV, it is possible that the poor reactivity to G1 is a reflection of the use of a heterologous antigen.

To determine whether antibodies to the SNV G1 antigen can decline over time in patients with well-documented SNV infection, a series of five paired serum samples from patients with PCR-confirmed SNV-associated HPS were examined in both the acute phase and a minimum of 3 months after recovery. These studies showed the complete loss of detectable antibodies to G1 in patients with previously detectable reactivity at 1, 1.5, and 1.8 years, although two other patients retained 4+ G1 peptide reactivity at 3 months and 1.25 years (data not shown). In none of these paired samples was a diminution of reactivity to N peptide or rN apparent.

DISCUSSION

Development of criteria for a positive assay result. Confirmatory tests for the diagnosis of viral infections such as Western blot assays and the RIBA SIAs for human immunodeficiency virus antibodies commonly detect isolated reactivities to individual antigens in low-risk populations (33). As a consequence, reactivity to multiple bands in specific patterns is generally required to make a positive diagnosis. Previous studies by SNV-based recombinant Western blot assays indicated that the requirement for reactivity to both SNV N and SNV G1 antigens was a useful criterion for a positive assay result (17, 23). For the purpose of developing a preliminary diagnostic criterion for SIA seropositivity to SNV, we considered that we would wish to detect as many of the earliest cases of SNV-associated HPS as possible, while avoiding incorrect interpretation of the inevitable isolated reactivity due to irrelevant cross-reactivity among negative controls (33).

Since the primary purpose of the RIBA SIA for hantavirus is to detect antibodies to SNV or SEOV, reactivity to either of the immunodominant SNV or SEOV rN antigens is considered essential. Any other additional reactivity, whether against a peptide or a recombinant antigen, would be sufficient to consider a sample positive. Reactivity to any other band(s) is considered indeterminate, and the absence of reactivity to any antigen is considered negative. By these criteria, 58 of 59 acute-phase serum samples from patients SNV-associated HPS, 4 of 4 serum samples from patients with non-SNV-associated HPS, 15 of 16 serum samples from patients with PUUV-associated HFRS, 13 of 17 serum samples from patients with SEOV-associated HFRS, and 3 of 7 serum samples from patients with HTNV-associated HFRS were positive, and the rest were indeterminate.

The pattern of reactivity among different groups of samples is informative. For example, those that exhibited reactivity only to the SEOV rN antigen did not occur in our control population, but they did occur in patients with SEOV- or HTNV-associated HFRS. No HPS patient whose serum lacked antibodies to SNV rN was identified at any stage of infection, suggesting that only patients whose sera exhibit reactivity to rN need be considered potential HPS patients. This early and consistent response to N antigen is consistent with many previous reports of the serologic response to hantavirus infection (9, 14, 23, 30, 31, 49, 50). Since all samples from patients with known HPS or HFRS demonstrated reactivity to either SNV or SEOV rN, it is possible that the nonreactive category will later be redefined to include samples that react to a band(s) other than that for SNV or SEOV rN antigen, but that decision will await additional studies.

Early diagnosis of HPS. If recognized early, a patient with HPS can be triaged to a facility with advanced tertiary care capabilities such as inverse ratio ventilation (inspiration much greater than expiration). Transport to a tertiary care facility with previous experience with HPS could be considered. Some such facilities are involved in a National Institute of Allergy and Infectious Diseases-sponsored placebo-controlled trial of the antiviral drug ribavirin (22, 32). Tertiary care centers can also evaluate potential salvage therapies such as extracorporeal membrane oxygenation. At the University of New Mexico Health Sciences Center, extracorporeal membrane oxygenation is credited with preventing the deaths of two HPS patients who manifested all of the clinical and laboratory criteria predictive of incipient death (29a, 32).

Since the interventions described above are available, we believe that the use of a rapid and reliable specific diagnostic test in the early stages of HPS might favorably affect the prognosis (Fig. 4). Although an SNV ELISA prepared at CDC has been distributed to a number of state health department laboratories, its use requires relatively expensive equipment (an ELISA reader), which makes it most suitable for larger laboratories with relatively sophisticated capabilities (11). An important reason for developing the hantavirus SIA was to allow for the reliable and rapid diagnosis of HPS at the earliest possible stage of illness, even in facilities with relatively unsophisticated laboratory capabilities. The SIA used in this study can be performed in any laboratory that has the equipment to measure liquids in volumes of 20 μl to 100 ml, an orbital shaker, and a platform rocker.

The performance of the hantavirus SIA for early diagnosis is promising, in that the test could detect antibodies in the earliest available serum samples from 100% of 59 patients with acute SNV-associated HPS. All but one (1.7%) of these early samples had reactivities that met the criteria for a positive assay result. For the patient whose single early HPS sample produced an indeterminate assay result, a serum sample was collected 3 days later, and by then the SIA result was strongly positive (Fig. 2, patient 3). Our study is limited, however, by the rarity of samples collected from patients in the earliest stages of illness. Samples collected during the prodromal phase of illness are almost never available, despite our aggressive attempts to prevent their disposal from laboratories.

In a control population, 2 samples that were judged to represent probable past hantavirus exposure were identified in a cohort of 192 samples. These samples showed IgG reactivities, and neither sample had detectable IgM reactivity by Western blot assay. Our results support a previously determined han-

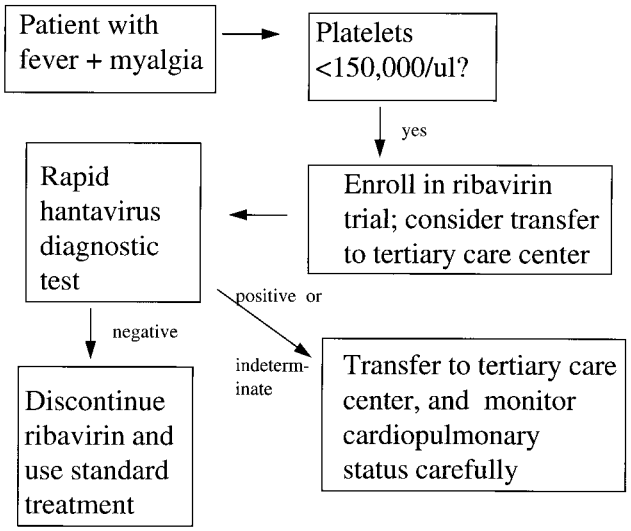

FIG. 4. Proposed algorithm for use of rapid hantavirus antibody testing in the placebo-controlled trial for ribavirin treatment of HPS. The trial is sponsored by the National Institute of Allergy and Infectious Diseases' Collaborative Antiviral Study Group. Enrollment sites include the University of New Mexico at Albuquerque, the University of Alabama at Birmingham, and the University of Utah at Salt Lake City. Patients are enrolled automatically if they have fever, myalgia, and thrombocytopenia. They can be removed from the ribavirin protocol if they are seronegative. Those not previously enrolled because of clinical signs and thrombocytopenia can become enrolled if they become seropositive.

tavirus seroprevalence of approximately 1% in adult American Indian populations of the southwestern United States (3). The epitope mapping studies that are inherent in the design of the SIA (by virtue of its use of the N peptide antigen) make it a potentially useful confirmatory assay in hantavirus seroprevalence studies.

False reactivities. We believe that the two control serum samples that reacted to both rN and N peptide antigens were positive due to past hantavirus infection. Two other serum samples reacted to rN in isolation, and both were reduced in intensity by preadsorption with *E. coli* lysates containing MBP, the fusion partner for SNV rN. Neither recognized a T7 gene 10–SNV N fusion protein in Western blot assays. We believe that these are probably reactive with trace contaminants from *E. coli* or perhaps to MBP. Four other serum samples were reactive with either peptide antigens or rG1. We do not believe that these sera are directed against a hantavirus, because there is at present no evidence to support the occurrence of human hantavirus infections that do not elicit antibodies to full-length N antigen (9, 14, 23, 30, 31, 49, 50).

Use of SIA in HPS due to hantaviruses other than SNV. The number of cases of HPS due to viruses other than SNV is too limited to draw conclusions about the performance of the hantavirus SIA for early diagnosis. However, the results reported here for patients with NYV-, BCCV-, and BAYV-associated HPS suggest that patients with an HPS-like syndrome who exhibit reactivity to rN and N peptide antigens in the absence of G1 peptide or rG1 antigens may be infected with a hantavirus other than SNV. The very high frequency of G1 peptide and rG1 reactivity among patients with SNV-associated HPS is thus a diagnostically useful finding. Recently, the absence of G1 reactivity in two patients with HPS in Texas led to the preliminary judgment that a hantavirus species other than SNV was involved, an observation that led to the identification of the second and third known human infections with BAYV (14a, 21). As new HPS cases continue to be recognized, it is likely that new etiologic agents will continue to be identified, and a rapid means for distinguishing new agents from SNV in a simple assay may help identify those infections.

TABLE 5. Amino acid identities between SNV N peptide, rN, G1 peptide, and rG1 antigens and the corresponding regions of other pathogenic hantaviruses

Virus	Prototype isolate	% Amino acid identity			
		SNV G1 peptide	SNV N peptide	SNV rN	SNV rG1
NYV	RI-1	83.3	93.0	93.8	86.6
BCCV	807040	58.0	86.0	82.6	61.0
BAYV	BAYV	61.3	95.3	86.0	59.8
PUUV	Sotkamo	45.2	74.4	64.5	47.6
SEOV	SR-11	45.2	48.8	49.6	26.8
HTNV	76-118	41.9	46.5	51.7	28.0

The G1 glycoprotein is the most variable protein of hantaviruses (Table 5), and it seemed appropriate to determine whether regional variation in the sequence of that portion of SNV would reduce the diagnostic utility of the G1 peptide or rG1 antigen. A region spanning the G1 epitope has been sequenced from a substantial number of *Peromyscus*-borne hantaviruses both from patients and from rodents (17, 20). A summary of sequencing studies of G1 genes obtained from patients with SNV and NYV is presented in Fig. 5. The primary sequence of most SNV G1 epitope-homologous segments is exactly that of the prototypical SNV isolate of New Mexico. One G1 gene sequence from Mono County, Calif., predicted a G1 epitope sequence that differed by 1 residue from that used in the SIA, and a the sequence of the virus from a patient in British Columbia differed by 3 amino acid residues from the sequence for the prototype SNV isolate. Although serum samples from these patients recognized the G1 peptide antigen of the SIA, a serum sample from an NYV-infected patient serum did not (Fig. 5). The sequence of the NYV G1 epitope differed by 5 residues from that of SNV (20).

A.

LKIESSCNFDLHVPATTTQKYNQVDWTKKSS	No. seqs.	Location(s)	G1 pep Reactivity
----------	21	8 states + Alberta	1+ to 4+
----------A----	1	California	2+
--------L------A----------N----	1	British Columbia	1+
----------S-SI------E-A----	1	New York	0

B.

-VP-----M-N-QSLS-IN-LTK-I-R--AN

FIG. 5. (A) Collected experience correlating primary amino acid sequence of the G1 antigen with reactivity against SNV G1 peptide. The amino acid sequence of the G1 peptide antigen of SNV (3H226) used on the hantavirus SIA membrane is shown. The sequence of the homologous peptides encoded by SNVs and NYVs from a variety of locations in the United States and Canada are depicted below the prototypic sequence. Residues that are identical to the 3H226 peptide are indicated with dashes. The number of genes encoding each peptide and their geographic derivation(s) are indicated, as are the intensities of reactivity observed against the G1 peptide. Only sequences derived from HPS patients are depicted. (B) The corresponding segment of SEOV SR-11.

Antibody classes. By using a conjugate against human heavy- and light-chain antibodies, the SIA is capable of detecting both IgG and IgM responses. This design could potentially increase the sensitivity of detection of antibody responses in the early stages of infection. However, since IgG and IgM responses are not distinguishable in the SIA, it is possible that past infections could cause false diagnoses of acute HPS.

Fortunately, serologic evidence of remote SNV infection appears to occur at a very low frequency (~1%) in control populations (3). Detection of these reactivities could potentially trigger more aggressive management of a patient with findings compatible with the HPS prodrome. However, the reactivity patterns exhibited by patients with remote SNV infection appear to be stereotyped and distinguishable from those exhibited by patients with acute SNV-associated HPS. The collective results of our studies of patients with well-documented past HPS, including those for whom early serum samples can be compared with convalescent-phase samples, indicate that G1 peptide and rG1 reactivities are usually lost in convalescence-phase serum samples (Table 2). Thus, we expect that it will often be possible to distinguish remote from acute SNV infection by reactivity to G1.

Use of SIA with serum samples from with patients with HFRS. The hantavirus SIA was designed to detect SNV antibodies. However, the rN antigen of SEOV was incorporated in the hantavirus SIA in response to reports of SEOV-associated HFRS and chronic renal disease in North America (12, 13). As expected, the sera from patients with SEOV-associated HFRS were universally reactive with the SEOV rN antigen, with variable reactivity to the SNV rN or N peptide antigen. Surprisingly, two patients with SEOV infection demonstrated 1+ reactivity against the G1 peptide of SNV, suggesting a possible conformational similarity in these two highly divergent antigens. Most of the sequence similarity between the SEOV and SNV G1 homologs are concentrated in the N terminus of the epitope (Fig. 5), so we suspect that this may be the most likely site for cross-reactive antibody recognition. The lack of reactivity to rG1 in these two serum samples could be due to a higher antigen density in the G1 peptide compared to that for the rG1 antigen or could be due to a nonviral epitope generated by conjugation of the G1 peptide to BSA. Another serum sample from a patient with SEOV-associated HFRS appears to have recognized an epitope in rG1 that is outside of the epitope represented in rG1. Since SEOV-associated HFRS patients and SNV-associated HPS patients are distinguishable by the reactivities of their sera to other antigens in the SIA, geographic origins, and clinical presentations, we do not anticipate that occasional G1 peptide reactivity in sera from SEOV-associated HFRS patients will represent a diagnostic problem.

No antigens of HTNV or PUUV are included in the hantavirus SIA. As expected, reactivity was somewhat weak for sera from patients with HTNV-associated HFRS and was primarily confined to the SEOV rN antigen, which bears the closest resemblance to the N antigen of HTNV. Among the sera from patients with PUUV-associated HFRS, the sera of all but one patient met the criterion for positivity (N peptide and SNV rN reactivities), with none of the sera exhibiting reactivity against the SNV G1 antigen. The serum of one patient had an indeterminate reactivity pattern. The relatively close relationship between the N antigens of PUUV and SNV presumably underlies the generally high frequency of detection of PUUV antibodies in the SIA.

ACKNOWLEDGMENTS

We are indebted to a large number of individuals at state and provincial health departments, the University of New Mexico, CDC, the Canadian Laboratory Centre for Disease Control, and hospitals for providing us with helpful advice, clinical specimens, and/or case histories. These include most especially M. Ascher, S. Banerjee, D. Dick, L. Grady, J. Greenblatt, D. Goade, C. Greenwalt, M. Jay, C. Kioski, F. Koster, T. Ksiazek, H. Levy, G. Mertz, D. Mosley, R. Moulton, S. Neill, G. Oty, C. J. Peters, J. Rawlings, R. Steece, and C. Ward.

This study supported by PHS grant RO1 AI 36336 and by the University of New Mexico Research Allocation Committee.

REFERENCES

1. **Avsic-Zupanc, T., S.-Y. Xiao, R. Stojanovic, A. Gligic, G. van der Groen, and J. W. LeDuc.** 1992. Characterization of Dobrava virus: a hantavirus from Slovenia. J. Med. Virol. **38:**132–137.
2. **Brummer-Korvenkontio, M., A. Vaheri, T. Hovi, C. H. con Bonsdorff, J. Vuorimies, T. Manni, K. Penttinen, N. Oker-Blom, and J. Lähdevirta.** 1980. Nephropathia epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. J. Infect. Dis. **141:**131–134.
3. **Centers for Disease Control and Prevention.** 1993. Update: outbreak of hantavirus infection—southwestern United States, 1993. Morbid. Mortal. Weekly Rep. **42:**441–443.
4. **Centers for Disease Control and Prevention.** 1994. Hantavirus pulmonary syndrome—United States, 1993. Morbid. Mortal. Weekly Rep. **43:**45–48.
5. **Centers for Disease Control and Prevention.** 1996. Hantavirus pulmonary syndrome—United States, 1995 and 1996. Morbid. Mortal. Weekly Rep. **45:** 291–295.
6. **Childs, J. E., T. G. Ksiazek, C. F. Spiropoulou, J. W. Krebs, S. Morzunov, G. O. Maupin, K. L. Gage, P. E. Rollin, J. Sarisky, R. E. Enscore, J. K. Frey, C. J. Peters, and S. T. Nichol.** 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary rodent reservoir for a new hantavirus in the southwestern United States. J. Infect. Dis. **169:**1271–1280.
7. **Chu, Y. K., H. W. Lee, J. W. LeDuc, C. S. Schmaljohn, and J. M. Dalrymple.** 1994. Serological relationships among viruses in the *Hantavirus* genus, family Bunyaviridae. Virology **198:**196–204.
8. **Duchin, J. S., F. T. Koster, C. J. Peters, G. L. Simpson, B. Tempest, S. R. Zaki, T. G. Ksiazek, P. Rollin, S. Nichol, E. Umland, R. L. Moolenaar, S. E. Reef, K. B. Nolte, M. M. Gallaher, J. C. Butler, R. F. Breiman, and the Hantavirus Study Group.** 1994. Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. N. Engl. J. Med. **330:**949–955.
9. **Elgh, F., G. Wadell, and P. Juto.** 1995. Comparison of the kinetics of Puumala virus specific IgM and IgG antibody responses in nephropathia epidemica as measured by a recombinant antigen-based enzyme-linked immunosorbent assay and an immunofluorescence test. J. Med. Virol. **45:**146–150.
10. **Elliott, L. H., T. G. Ksiazek, P. E. Rollin, C. F. Spiropoulou, S. P. Morzunov, M. Monroe, C. S. Goldsmith, C. D. Humphrey, S. R. Zaki, J. W. Krebs, G. Maupin, K. Gage, J. E. Childs, S. T. Nichol, and C. J. Peters.** 1994. Isolation of the causative agent of hantavirus pulmonary syndrome. Am. J. Trop. Med. Hyg. **51:**102–108.
11. **Feldmann, H., A. Sanchez, S. Morzunov, C. F. Spiropoulou, P. E. Rollin, T. G. Ksiazek, C. J. Peters, and S. T. Nichol.** 1993. Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. Virus Res. **30:**351–367.
12. **Glass, G. E., A. J. Watson, J. W. LeDuc, G. D. Kelen, T. C. Quinn, and J. E. Childs.** 1993. Infection with a ratborne hantavirus in U.S. residents is consistently associated with hypertensive renal disease. J. Infect. Dis. **167:**614–620.
13. **Glass, G. E., A. J. Watson, J. W. LeDuc, and J. E. Childs.** 1994. Domestic cases of hemorrhagic fever with renal syndrome in the United States. Nephron **68:**48–51.
14. **Gött, P., L. Zöller, S. Yang, R. Stohwasser, E. K. F. Bautz, and G. Darai.** 1991. Antigenicity of hantavirus nucleocapsid proteins expressed in *E. coli*. Virus Res. **19:**1–16.

14a. **Hjelle, B., and J. Rawlings.** Unpublished data.

14b. **Hjelle, B., and N. Torrez-Martinez.** Unpublished data.

15. **Hjelle, B., R. F. Khabbaz, G. A. Conway, C. North, D. Green, and J. E. Kaplan.** 1994. Prevalence of HTLV-II in HTLV-II-endemic populations of the southwestern United States. Am. J. Trop. Med. Hyg. **51:**11–15.
16. **Hjelle, B., S. Jenison, N. Torrez-Martinez, T. Yamada, K. Nolte, R. Zumwalt, K. MacInnes, and G. Myers.** 1994. A novel hantavirus associated with an outbreak of fatal respiratory disease in the southwestern United States: evolutionary relationships to known hantaviruses. J. Virol. **68:**592–596.
17. **Hjelle, B., F. Chavez-Giles, N. Torrez-Martinez, T. Yamada, J. Sarisky, M. Ascher, and S. Jenison.** 1994. Dominant glycoprotein epitope of Four Corners hantavirus is conserved across a wide geographical area. J. Gen. Virol. **75:**2881–2888.
18. **Hjelle, B., C. F. Spiropoulou, N. Torrez-Martinez, S. Morzunov, C. J. Peters, and S. T. Nichol.** 1994. Detection of Muerto Canyon virus RNA in peripheral blood mononuclear cells from patients with hantavirus pulmonary syndrome. J. Infect. Dis. **170:**1013–1017.

19. **Hjelle, B., J. Krolikowski, N. Torrez-Martinez, F. Chavez-Giles, C. Vanner, and E. Laposata.** 1995. Phylogenetically distinct hantavirus implicated in a case of hantavirus pulmonary syndrome in the northeastern United States. J. Med. Virol. **46:**21–27.
20. **Hjelle, B., S.-W. Lee, W. Song, N. Torrez-Martinez, J.-W. Song, R. Yanagihara, I. Gavrilovskaya, and E. R. Mackow.** 1995. Molecular linkage of hantavirus pulmonary syndrome to the white-footed mouse, *Peromyscus leucopus*: genetic characterization of the M genome of New York virus. J. Virol. **69:**8137–8141.
21. **Hjelle, B., D. Goade, N. Torrez-Martinez, M. Lang-Williams, J. Kim, R. L. Harris, and J. A. Rawlings.** 1996. Hantavirus pulmonary syndrome, renal insufficiency and myositis associated with infection by Bayou hantavirus. Clin. Infect. Dis. **23:**495–500.

21a. **Hjelle, B., et al.** Unpublished data.

22. **Huggins, J. W., C. M. Hsiang, T. M. Cosgriff, M. Y. Guang, J. I. Smith, Z. O. Wu, J. W. LeDuc, Z. M. Zheng, J. M. Meegan, Q. N. Wang, D. D. Oland, X. E. Gui, P. H. Gibbs, G. H. Yuan, and T. M. Zhang.** 1991. Prospective, double-blind, concurrent, placebo-controlled clinical trial of intravenous ribavirin therapy for hemorrhagic fever with renal syndrome. J. Infect. Dis. **164:**119–127.
23. **Jenison, S., T. Yamada, C. Morris, B. Anderson, N. Torrez-Martinez, N. Keller, and B. Hjelle.** 1994. Characterization of human antibody responses to Four Corners hantavirus infections among patients with hantavirus pulmonary syndrome. J. Virol. **68:**3000–3006.
24. **Khan, A. S., C. F. Spiropoulou, S. Morzunov, S. R. Zaki, M. A. Kohn, S. R. Nawas, L. McFarland, and S. T. Nichol.** 1995. Fatal illness associated with a new hantavirus in Louisiana. J. Med. Virol. **46:**281–286.
25. **Khan, A. S., J. M. Gaviria, P. E. Rollin, W. G. Hlady, T. G. Ksiazek, L. R. Armstrong, R. Greenman, E. Ravkov, M. Kolber, H. Anapol, E. D. Sfakianaki, S. T. Nichol, C. J. Peters, and R. F. Khabbaz.** 1996. Hantavirus pulmonary syndrome in Florida: association with the newly identified Black Creek Canal virus. Am. J. Med. **100:**46–48.
26. **Lee, H. W., P.-W. Lee, and K. M. Johnson.** 1978. Isolation of the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. **137:**298–308.
27. **Lee, H. W., L. J. Baek, and K. M. Johnson.** 1982. Isolation of Hantaan virus, the etiologic agent of Korean hemorrhagic fever from wild urban rats. J. Infect. Dis. **146:**638–644.
28. **Lee, H. W., and K. M. Johnson.** 1982. Laboratory-acquired infections with Hantaan virus, the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. **146:**645–651.
29. **Lee, H. W., P. W. Lee, L. J. Baek, and Y. K. Chu.** 1990. Geographical distribution of hemorrhagic fever with renal syndrome and hantaviruses. Arch. Virol. Suppl. **1:**5–18.

29a. **Levy, H.** Personal communication.

30. **Lundkvist, Å., S. Björsten, and B. Niklasson.** 1993. Immunoglobulin subclass G responses against the structural components of Puumala virus. J. Clin. Microbiol. **31:**368–372.
31. **Lundkvist, Å., S. Björsten, B. Niklasson, and N. Ahlborg.** 1995. Mapping of B-cell determinants in the nucleocapsid protein of Puumala virus: definition of epitopes specific for acute immunoglobulin G recognition in humans. Clin. Diagn. Lab. Immunol. **2:**82–86.
32. **Mertz, G. J., B. L. Hjelle, and R. T. Bryan.** 1996. Hantavirus infection. Adv. Intern. Med. **42:**373–425.
33. **Midthun, K., L. Garrison, M. L. Clements, H. Farzadegan, B. Fernie, and T. Quinn.** 1990. Frequency of indeterminate Western blot tests in healthy adults at low risk for human immunodeficiency virus infection. J. Infect. Dis. **162:**1379–1382.
34. **Morzunov, S. P., H. Feldmann, C. F. Spiropoulou, V. A. Semenova, P. E. Rollin, T. G. Ksiazek, C. J. Peters, and S. T. Nichol.** 1995. A newly recognized virus associated with a fatal case of hantavirus pulmonary syndrome in Louisiana. J. Virol. **69:**1980–1983.
35. **Nerurkar, V. R., K. J. Song, J.-W. Song, J. W. Nagle, B. Hjelle, S. Jenison, and R. Yanagihara.** 1994. Genetic evidence for a hantavirus enzootic in deer mice (*Peromyscus maniculatus*) a decade before the recognition of hantavirus pulmonary syndrome. Virology **204:**563–568.
36. **Nichol, S. T., C. F. Spiropoulou, S. Morzunov, P. E. Rollin, T. G. Ksiazek, H. Feldmann, A. Sanchez, J. Childs, S. Zaki, and C. J. Peters.** 1993. Genetic identification of a novel hantavirus associated with an outbreak of acute respiratory illness in the southwestern United States. Science **262:**914–917.
37. **Pichuantes, S., L. M. Babe, P. J. Barr, and C. S. Craik.** 1989. Recombinant HIV1 protease secreted by *Saccharomyces cerevisiae* correctly processes myristylated gag polyprotein. Proteins Struct. Funct. Genet. **6:**324–327.
38. **Pichuantes, S., L. M. Babe, P. J. Barr, D. L. DeCamp, and C. S. Craik.** 1990. Recombinant HIV2 protease processes HIV1 $Pr53^{gag}$ and analogous junction peptides *in vitro*. J. Biol. Chem. **265:**13890–13898.
39. **Pichuantes, S., A. T. Nguyen, and A. Franzusoff.** 1996. Expression of heterologous gene products in yeast, p. 129–161. *In* J. L. Cleland and C. S. Craik (ed.), Protein engineering, principles and practice. Wiley-Liss Inc., New York, N.Y.
40. **Rollin, P. E., T. G. Ksiazek, L. H. Elliott, E. V. Ravkov, M. L. Martin, S. Morzunov, W. M. Livingstone, M. Monroe, G. Glass, S. Ruo, A. S. Khan, J. E. Childs, S. T. Nichol, and C. J. Peters.** 1995. Isolation of Black Creek Canal virus, a new hantavirus from *Sigmodon hispidus* in Florida. J. Med. Virol. **46:**35–39.
41. **Schmaljohn, A. L., D. Li, D. L. Negley, D. S. Bressler, M. J. Turell, G. W. Korch, M. S. Ascher, and C. S. Schmaljohn.** 1995. Isolation and initial characterization of a newfound hantavirus from California. Virology **206:**963–972.
42. **Sheshberadaran, H., B. Niklasson, and E. Tkachenko.** 1988. Antigenic relationship between hantaviruses analysed by immunoprecipitation. J. Gen. Virol. **69:**2645–2651.
43. **Song, J.-W., L. J. Baek, D. C. Gajdusek, R. Yanagihara, I. Gavrilovskaya, B. J. Luft, E. R. Mackow, and B. Hjelle.** 1994. Isolation of pathogenic hantavirus from white footed mouse (*Peromyscus leucopus*). Lancet **344:**1637.
44. **Steven, C., M. Johnson, and A. Bell.** 1994. First reported cases of hantavirus pulmonary syndrome in Canada. Can. Communic. Dis. Rep. **20:**121–125.
45. **Tomiyama, T., and H. W. Lee.** 1990. Rapid serodiagnosis of hantavirus infections using high density particle agglutination. Arch. Virol. Suppl. **1:**29–33.

45a. **Torrez-Martinez, N., and B. Hjelle.** Unpublished data.

46. **Werker, D. H., A. E. Singh, H. Artsob, and J. Hockin.** 1995. Hantavirus pulmonary syndrome in Canada. Can. Communic. Dis. Rep. **21:**77–80.
47. **Wilson, C., B. Hjelle, and S. Jenison.** 1994. A probable case of hantavirus pulmonary syndrome that occurred in New Mexico in 1975. Ann. Intern. Med. **120:**813.
48. **Yamada, T., B. Hjelle, R. Lanzi, C. Morris, B. Anderson, and S. Jenison.** 1995. Antibody responses to Four Corners hantavirus in the deer mouse (*Peromyscus maniculatus*): identification of an immunodominant region of the nucleocapsid protein. J. Virol. **69:**1939–1943.
49. **Zöller, L., J. Scholz, R. Stohwasser, L. B. Giebel, K. K. Sethi, E. K. F. Bautz, and G. Darai.** 1989. Immunoblot analysis of the serologic response in hantavirus infections. J. Med. Virol. **27:**231–237.
50. **Zöller, L., S. Yang, P. Gött, E. K. F. Bautz, and G. Darai.** 1993. A novel μ-capture enzyme-linked immunosorbent assay based on recombinant proteins for sensitive and specific diagnosis of hemorrhagic fever with renal syndrome. J. Clin. Microbiol. **31:**1194–1199.