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Generation of an HFRS Patient-Derived Neutralizing Recombinant Antibody to Hantaan Virus G1 Protein and Definition of the Neutralizing Domain

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Hantaan virus (HTNV) in the Hantavirus genus, family Bunyaviridae, is the major cause of severe hemorrhagic fever with renal syndrome (HFRS). We prepared a combinatorial phage display library of human Fabs to HTNV from RNA extracted from the blood lymphocytes of a convalescent HFRS patient. We selected two G1 glycoprotein-specific clones and one nucleocapsid protein (N)-specific clone from the Fab library for further studies. The human Fab antibodies were converted to IgG form in baculovirus/insect cells system by using cassette vectors that we developed earlier. Characterization of the recombinant antibodies revealed that the two G1-specific IgGs, could bind to and neutralize HTNV but not Seoul virus (SEOV). The N-specific IgG did not neutralize either HTNV or SEOV. Sequence analysis revealed that the two G1-specific clones differed by only one predicted amino acid in their complementarity determining regions, CDR3. Epitope mapping studies were carried out with one of the two G1-specific clones and synthetic peptides representing portions of HTNV G1. Results indicated that the recombinant antibody recognizes the core amino acid sequence LTKTLVIGQ, which is found near the C-terminus of HTNV G1. These results are the first to define a neutralizing epitope on the G1 protein of HTNV using an antibody derived from an HFRS patient.
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KEY WORDS: phage display; human recombinant antibody; Hantaan virus; G1 protein; epitope mapping

INTRODUCTION

Hantaviruses cause two life threatening diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). At least four hantaviruses cause HFRS: Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV), and Puumala virus (PUUV) [Schmaljohn and Hjelle, 1997]. The viruses are found throughout Eurasia, with China having the greatest number of cases [Hooper and Li, 2001]. HTNV is responsible for the majority of the severe HFRS cases occurring in China.

Protective immunity to hantaviral infections is thought to correlate with neutralizing antibody responses to the viral G1 and G2 envelope glycoproteins [Arikawa et al., 1989, 1992]. Most of the antigenic determinants on G1 and G2 of HFRS-causing hantaviruses are believed to be conformational, as reflected by the high cysteine content of both proteins and the poor reactivity of HFRS patient sera with denatured G1 or G2. Only a few epitope mapping studies of hantaviral envelope proteins have been reported. A competitive binding study of 24 mouse monoclonal antibodies (Mabs) revealed at least nine distinct, partially overlapping antigenic sites on HTNV G1 and G2 [Arikawa et al., 1989]. In subsequent studies with the same Mabs, sequence analysis of the genomes of Mab escape mutants revealed that noncontiguous amino acids form neutralizing epitopes (i.e., conformational epitopes) on the envelope proteins [Wang et al., 1993; Kikuchi et al., 1998]. Conformational, neutralizing epitopes were also identified on PUUV using phage display to express

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random peptides and by reacting the expression products with two G2-specific Mabs to PUUV [Salonen et al., 1998, Heiskanen et al., 1999, Nicacio et al., 2000]. Thus, evidence to date suggests that neutralizing epitopes of the G1 and G2 proteins of HFRS-causing hantaviruses are frequently conformational.

We describe selection and characterization of a neutralizing human IgG antibody to HTNV G1 derived from RNA extracted from lymphocytes of a convalescent HFRS patient by using phage display and recombinant antibody technology. In addition we report, for the first time, fine epitope mapping of a human neutralizing antibody to the G1 protein of HTNV and identify its antigen-binding domain.

MATERIALS AND METHODS

Viruses, Cells, Lymphocytes, and Mabs

The strains of HTNV and SEOV used in this study were described previously and include HTNV strains 76–118, A9, 84Fli, Chen, H5 and SEOV strains HR-80–39, L99, R22, HB55 [Liang et al., 1994]. Viruses were propagated in Vero-E6 cells (ATCC, Rockville, MD; C1008 CRL 1586). The human lymphocytes used for RNA extraction were isolated from a HFRS patient from Anhui Province, China, during a 2-month convalescent phase. The mouse Mabs to HTNV G1 (6D4) and G2 (11E10) were described earlier [Arikawa et al., 1989].

Construction, Panning, and Screening of a Human Antibody Phage-Display Library

The HFRS patient blood lymphocytes were purified on a Ficoll gradient. Total cellular RNA was extracted from pelleted lymphocytes by using Trizol reagent (GIBCO BRL, Gaithersburg, MD) and cDNA was synthesized by using oligo (dT) primers and reverse transcriptase. The heavy chain Fd and light chain genes were amplified from the cDNA by polymerase chain reaction (PCR) and cloned sequentially into the phagemid vector pComb3 as described elsewhere [Barbas et al., 1991] The human antibody phage library was constructed after transformation of *E. coli* followed by the rescue of helper phage as described previously [Barbas et al., 1991]. The antibody phage library was selected by 3–4 rounds of alternative panning on purified HTNV virions [Schmaljohn et al., 1983] or on HTNV glycoproteins captured with mouse Mabs. Briefly, for the first round of panning, 6–8 wells of a 96-well immunoplate were coated with 0.5–1 μg/well of purified HTNV (strain 76–118) overnight at 4°C. For the second round of pannings, the plates were first coated with 0.5–1 μg/well of a mixture of Mabs 6D4 (G1-specific) and 11E10 (G2-specific), followed by addition of a lysate of HTNV-infected Vero-E6 cells. The lysate was prepared by treating Vero-E6 cells infected for 7–8 days with HTNV with 2% NP-40. Nuclei were removed from the lysates by centrifugation and the lysate was diluted with 0.02 M phosphate buffered saline (PBS) containing 4% skim milk. Lysate (100 μl) was added to the antibody coated plates and plates were incubated at 37°C for 2 hr. The plates were then washed with 0.02 M PBS containing 0.05% Tween-20 and blocked with PBS containing 4% skim milk, then 100 μl of the phage libraries was added to the plates and panning was continued as described previously [Barbas et al., 1991]. After 3 or 4 rounds of panning, the human Fab antibody genes were expressed in *E. coli* and the Fab were screened by ELISA and an immune fluorescence assay (IFA) as described below.

Sequence Analysis of the Variable Regions of the Fabs

Nucleic acid sequencing was carried out with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Inc., Boston, MA) and the primers: 5′CCGCGGTGGCGGCCGCAA3′ for the VH gene of the heavy chains and the primer 5′AAACTAGCTAGTCGCCAAGGA3′ for the VL of the light chains. Sequences were analyzed by aligning to human immunoglobulin sequences present in GenBank and the CDR regions were determined with the V-Base program (Center for Protein Engineering, Cambridge, UK).

Construction and Expression of Complete IgG

The VH and VL or Fab genes of three of the human Fabs selected from the phage library were cloned into IgG expression cassette vectors pAc-L-CH3, pAc-K-CH3 or pAc-L-Fc, as described previously, such that in-frame ligations to a human Fc region were obtained [Liang et al., 2001]. The resulting expression vector DNA containing heavy and light chain genes were transfected into SF9 insect cells, and recombinant baculoviruses were prepared by homologous recombination using the Baculo-Gold transfection kit (Pharmingen, San Diego, CA) according to the manufacturer's instructions. To assay expression, IFA was carried out on SF9 cells infected with plaque purified recombinant baculoviruses using FITC conjugated anti-human Fc-specific antibodies. Secreted, recombinant human IgG antibodies were detected in the supernatants of infected SF9 cells by a conventional capture ELISA, using goat anti-human IgG Fab as a capture reagent and HRP conjugated anti-human Fc for detection.

ELISA

ELISA were carried out to screen crude Fab or IgG antibody preparations, determine the binding avidities of the Fab or IgG antibodies to hantaviruses, and for antigenic analysis of Fab or IgG antibodies to various hantaviruses. Direct ELISA was carried out by coating each well of a 96-well plate with approximately 0.5–1 μg of sucrose gradient purified HTNV diluted in 0.01 M sodium carbonate buffer, pH.8.6. The plates were blocked with 4% skim milk in PBS, after which the crude or purified Fab or IgG antibody preparations were added. Wells were washed and incubated with HRP-conjugated anti-human Fab or anti-human Fc (Sigma, St. Louis, MO) followed by TMB substrate for color development. Antibody capture ELISA was carried out by coating

plates with approximately 0.5 μg/well, of mouse Mabs to HTNV G1 or G2(6D4 or 11E10) or N (L13F3). The plates were washed three times with PBS-0.02% Tween, followed by infected cell lysates prepared by treatment with NP40 as described above. After washing, the test Fab or IgG antibody preparations were added to the wells and subsequent steps were the same as those described for the direct ELISA.

Immunofluorescence Assays (IFA)

The HTNV, SEOV, and PUUV IFA antigen slides were purchased from Progen (Heidelberg, Germany) or were prepared in our laboratory using standard conditions [Lee et al., 1999]. Briefly, Vero-E6 cells were infected with hantavirus for 7–14 days. The infected cells were digested with trypsin-EDTA (GIBCO-BRL) and washed with PBS once, then resuspended in PBS at the concentration around 1×10^6 cells/ml. The cells were fixed on 10- or 12-well slides with cold acetone at −20°C for at least 30 min, then were dried and kept at −70°C for next steps. DOBV antigen slides were kindly provided by Dr. T. Avsic-Zupanc, Institute of Microbiology and Immunology, Lubjana, Slovenia. In addition to these slides prepared with authentic hantaviral antigens, for HTNV and SEOV screening we prepared IFA slides from recombinant baculovirus-infected SF9 cells. Briefly, SF9 cells cultured in T25 flasks were infected with recombinant viruses expressing HTNV or SEOV M segments for 3–4 days. The infected cells were washed and resuspended in PBS then fixed on 10- or 12-well slides with acetone at room temperature for 10 min. Cell culture supernatants of SF9 cells infected with recombinant baculoviruses expressing human IgG antibodies to G1 and NP were incubated with the slides described above at 37°C for 30 min. Bound antibodies were detected with FITC conjugated anti-human Fab and human Fc antibodies.

Radioimmunoprecipitation of HTNV with the Recombinant Antibodies

Confluent monolayers of Vero-E6 cells in T25 flasks were infected with 2 ml (0.5×10^6 PFU) of HTNV strain 76–118. Twenty-four hours postinfection, the cell culture medium was removed and replaced with cysteine-and methionine-free Eagles' Minimal Essential medium containing 2% FBS, and incubated for 1 hr at 37°C. Cells were radiolabeled with 200 μCi of 35S Promix (Amersham, Buckinghamshire, UK) per flask for 4 hr. The cells were lysed on ice with 1 ml of cell lysis buffer composed of pH 8.0 TNE, then centrifuged to remove the nuclei. The radiolabeled HTNV proteins were immunoprecipitated by mixing 100 μl of cell lysate with 100 μl of a HFRS patient serum or the supernatants of SF9 cells infected with recombinant baculoviruses expressing human antibodies. After incubation at 4°C overnight, protein G-Sepharose 4B (Sigma) was added and the mixture was shaken at 4°C for 3 hr. The beads with immunoprecipitated proteins were washed 3 times with lysis buffer containing 1% Zwittergent and once with 0.1 M Tris-HCl, 1 mM EDTA, then resuspended in 2× regular SDS-PAGE loading buffer and analyzed by SDS-PAGE as described previously [Schmaljohn et al., 1983].

Preparation and Purification of IgG Antibodies

The methods for the preparation and purification of recombinant baculoviruses expressing human IgG antibodies has been described in detail [Liang et al., 2001]. Briefly, supernatants from the recombinant baculovirus-infected SF9 or H5 insect cells were clarified and applied to Protein-G Sepharose 4B affinity columns (Sigma Chemical Co., St. Louis, MO), washed, eluted, neutralized and finally desalted by passing through a desalting column. IgG concentrations were detected by UV spectrophotometer and calculated according to the equations of protein quantitation from Harlow and Lane [1988] and adjusted to a concentration of 200–500 μg/ml. Under reducing and nonreducing conditions 2 μg of purified IgG antibody was examined by SDS-PAGE as described previously [Liang et al., 2001].

Plaque Reduction Neutralization Test (PRNT)

PRNTs for HTNV and SEOV were carried out using methods described previously [Schmaljohn et al., 1983]. Briefly, Vero-E6 cells were cultured in 24-well plates for one week. The purified human recombinant Fab and IgG antibodies were standardized to 100 μg/ml and diluted serially in two-fold increments. Aliquots of each antibody dilution (100 μl each) were incubated with 100 PFU of HTNV or SEOV at 4°C overnight. Virus-antibody mixtures were applied to the cell monolayers and incubated at 37°C for 1 hr, then overlaid with 0.6% agarose-EMEM. After incubation at 37°C, 5% CO_2 for 7–8 days, an overlay of 0.6% agarose-neutral red EMEM was added to the plates. The percent neutralization of antibodies was calculated by comparing the maximum average plaque numbers from the virus control and the plaque numbers from the antibody dilutions. Neutralizing activity was determined by 50% reduction of plaques as compared to the control.

Peptide Scan of the Epitope of Human Recombinant Antibody IgG100

Peptides (15mers) covering the entire amino acid sequence of G1 of HTNV (strain 76–118, GenBank accession number M14627) were synthesized with a ASP222 machine on activated membranes using fMOc protected amino acid derivatives (Sigma–Genosys, Cambridgeshire, UK) according to the procedure described previously [Frank, 1992]. An immunoassay was carried out using a protocol published previously [Bluthner et al., 2000], with some modifications. Briefly, the membranes with synthesized peptides on them were washed three times for 10 min in TBS buffer (10mM Tris/HCl, pH 7.6, 150 mM NaCl). Nonspecific binding sites were blocked by overnight incubation in TBS-2% milk at 4°C. The membranes were washed once with

TBS-0.2% Tween-20 buffer, and were incubated with antibody dilutions (5 μ/ml) in TBS-2%-powdered skim milk for 2 hr. The unbound antibodies were removed by washing the membrane three times with TBS-T, and afterwards three times in TBS for 5 min per wash. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody was added and the membranes incubated at room temperature, then washed as above. ECL Western blotting detection reagents were used to visualize the proteins (Amersham).

Fine Mapping of IgG 100 by Mutational Analysis and Structure Prediction

The major epitope containing the peptide GQRKVIL-TKTLVIGQ was analyzed by glycine-walking. Each amino acid except the glycine in the peptides was replaced by glycine individually, and the glycine was replaced by alanine. The resulting mutated peptides were incubated with the human IgG antibody IgG100 and analyzed as described above. Secondary structure prediction of the peptide was carried out with the program PEPTIDE STRUCTURE from the HUSAR package (Version 5) of the German Cancer Research Center (Heidelberg, Germany).

RESULTS

Selection and Initial Characterization of HTNV-Specific Fab

A combinatorial antibody library was constructed from RNA extracted from the blood lymphocytes of a convalescent HFRS patient exhibiting high levels of HTNV-specific antibodies (data not shown). PCR-amplified human heavy and light chain Fab genes were inserted into the phagemid vector pComb3 and the Fabs were displayed on the surface of VCS M13 filamentous phage. The library was panned and screened against purified HTNV or viral glycoproteins selected from an infected cell lysate by antibody capture. Fab antibody clones taken from 3–4 rounds of panning were chosen for further study by their reactivity in ELISA. Ten Fab clones were selected and characterized for their specific binding properties and genetic information (Table I). The Fab preparations were screened by ELISA using plates coated with purified HTNV, or with G or N captured with specific Mabs, as described in the Methods section. All 10 Fab clones reacted with purified HTNV; of these, eight clones (AH7, AH8, AH23, AH55, AH56, AH95, AH100, and AH105) recognized HTNV glycoproteins and two clones (AH30, AH34) recognized NP in the various ELISA (Table I). Sequence analysis of the 10 Fab clones showed the glycoprotein-specific Fab to be very similar except AH 23 showed 1 amino acid change with others in the VL CDR1 regions. All of them were composed of IgG1 Fd heavy chains and lambda light chains. Similarly, the two N-specific clones (AH30, AH34) had nucleotide sequences encoding IgG1 Fd heavy chains and kappa light chains that were very similar (Table I). Moreover, the deduced amino acid sequences of the heavy and light chain complementarity-determining region, CDR3, which is known to be the most variable region in antibody molecules, were nearly identical for the Fabs reactive with glycoproteins. Likewise, the CDR3 of the two N-reactive Fabs were nearly identical. Therefore, it is likely that the G and N Fab clones were derived from only two parent clones.

Linkage of Selected Fab Genes to Human IgG Fc and Expression by Recombinant Baculoviruses

According to the sequences and the antigen binding properties of the above Fab clones, the variable heavy (VH) and light (VL) genes of Fab clones AH100 (G_1-reactive), and AH30 (N-reactive), and the Fd and

TABLE I. Characteristics of Fab Clones Selected From Phage Display Antibody Libraries

		ELISA (OD_{490})					
Fab clones	IFA[a]	Virion[b]	N[c]	G1 + G2[d]	PRNT (%)[e]	CDR3 (VH)[f]	CDR3 (VL)[f]
AH7	+	0.45	0.02	0.53	<10	YYCARGDSSGYYPLNWFG	YYCETWDSSLEL–WYFGG
AH8	+	0.56	0.01	0.67	25	YYCARGDSSGYYPLNWFG	YYCETWDSSLSGLWYFGG
AH23	++	0.89	0.02	0.82	45	YYCARGDSSGYYPLNWFG	YYCETWDSSLECLWYFGG
AH30	++++	0.68	1.02	0.02	<10	YYCARGHEHWSILDYWFG	YICQHFGRSQYTFGG
AH34	++++	0.72	0.99	0.00	<10	YYCARGHEHWSILDYWFG	YICQQFGRSQYTFGG
AH55	+	0.48	0.01	0.53	35	YYCARGDSSGYNPLNWFG	YFCGTWDKSLECLWYFGG
AH56	+	0.59	0.00	0.45	<10	YYCARGDSSGYNPLNWFG	YFCGTWDKSLECLWYFGG
AH95	++	0.93	0.01	0.86	38	YYCARGDSSGYNPLNWFG	YYCETWDSSLECLWYFGG
AH100	++	0.97	0.00	0.92	55	YYCARGDSSGYNPLNWFG	YYCETWDSSLECLWYFGG
AH105	+	0.93	0.05	0.95	34	YYCARGDSSGYNPLNWFG	YYCETWDSSLECLWYFGG

[a]IFA: Immunofluorescence assays were performed on HTNV-infected Vero E6 cells as described in Methods, (+) to (++++) indicates the relative intensity of fluorescence.
[b]Virion, Sucrose gradient purified HTNV.
[c]Capture ELISA performed with HTNV N antigen from infected Vero-E6 cells.
[d]Capture ELISA performed with HTNV G1 and G2 antigen from infected Vero-E6 cells.
[e]PRNT values represent the average percent reduction in number of plaques compared to a negative control.
[f]Partial amino acid sequences of the VH and VL regions of 10 Fab clones, the CDR3 region of VH and VL were indicated as underlined.

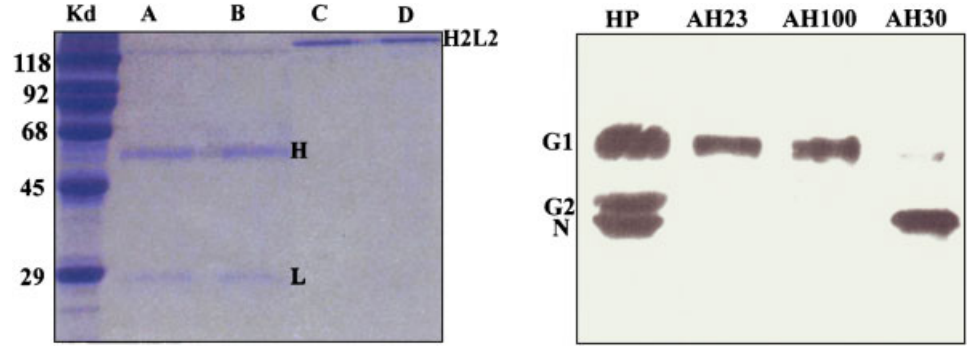

Fig. 1. SDS-PAGE of affinity-purified recombinant human IgG antibodies to HTNV. **A:** AH100IgG (lanes A,C) and AH23IgG (lanes B,D) were purified from recombinant baculovirus infected insect cells by affinity chromatography. About 0.8–1 μg of each antibody was analyzed by gel electrophoresis under reducing (lanes A,B) or nonreducing (lanes C,D) conditions. Proteins were visualized by staining with Coomassie blue. H2L2, heterodimer of 2 heavy and 2 light chains; H, heavy chain; L, light chain. **B:** Radiolabeled HTNV proteins were immunoprecipitated with HFRS patient serum (lane HP) or with recombinant human antibodies as indicated above each lane. The positions of the viral envelope glycoproteins, G1 and G2, and the nucleocapsid protein, N, are indicated to the left of the autoradiogram.

light chain genes of Fab clone AH23 (G_1-reactive) were selected and cloned individually into IgG baculovirus-expression cassette vectors pAc-L-CH3, pAc-K-CH3 (designed for cloning of scFv gene)and pAc-L-Fd (designed for cloning Fab genes) as described previously [Liang et al., 2001] Recombinant baculoviruses were prepared by homologous recombination using the Baculo-Gold transfection kit (Pharmingen) according to the instructions given by the supplier. The human recombinant intact antibodies to HTNV G1 (AH100IgG and AH23IgG) and N (Ah30IgG) were expressed in SF9or High 5 (H5) insect cells after the infection of the recombinant baculoviruses. The expressed IgG antibodies were purified and concentrated to 150–200 μg/ml. SDS-PAGE analysis of AH100IgG (IgGλ) and AG30IgG (IgGκ) revealed that antibody products expressed by the baculovirus/insect cells were of the expected sizes for the complete heavy and light chains of IgG under reducing (about 50 kD and 25 kD) and nonreducing (150 kD) conditions (Fig. 1A). AH23IgG showed the same electrophoresis mobility under above two conditions (Data not shown). These results demonstrate that the baculovirus-expressed, recombinant human IgG antibodies produced in insect cells were correctly assembled and secreted as authentic heterodimeric immunoglobulins.

Characterization of Selected Human Recombinant Antibodies to HTNV

To identify the binding specificity of the human recombinant antibodies, clarified supernatants from recombinant baculovirus-infected SF9 cells were assayed by immunoprecipitation, and IFA. The human antibodies AH100IgG and AH23IgG immunoprecipitated radiolabeled HTNV G1 and AH30IgG precipitated HTNV N (Fig. 1B). IFA was carried out with AH100IgG on HTNV-infected Vero-E6 cells or insect cells infected with the recombinant baculovirus expressing HTNV glycoproteins [Shi et al., 1995] confirmed the HTNV binding specificities (Fig. 2). To investigate whether the G1 specific human antibodies (AH 100IgG and AH23IgG) bind to only HTNV or are cross-reactive with other HFRS-causing hantaviruses, IFA, indirect ELISA and PRNT were carried out. In these three types of assays, AH100IgG reacted only with HTNV, and not with SEOV, DOBV, or PUUV (Table II). As expected, AH23IgG had nearly the same binding specificity and neutralizing ability as AH100 IgG (data not shown). These results confirm that the single amino acid difference in the VH CDR3 regions of these two antibodies does not influence their function (Table I). Because of these findings, we used only AH100 for the remainder of our studies.

To further evaluate the neutralizing activity and the binding affinity of AH100IgG, we carried out PRNT with two HTNV strains, 76–118 and 84Fli, and two SEOV strains, HR80–90 and L99. AH100 IgG clearly neutralized both HTNV strains. Approximately 1.25 μg of the affinity purified IgG was required to achieve 80% reduction in plaques, and 0.3–0.6 μg was required to achieve 50% plaque reduction (Fig. 3). Consistent with the IFA and ELISA results, AH100 did not neutralize either strain of SEOV. As expected, AH30IgG to HTNV N did not neutralize either HTNV or SEOV. Further, indirect ELISA as described in the methods measured the binding affinity of AH100IgG human antibody. Approximately 125 ng (about 8×10^{-8} M) of purified AH100IgG was required to yield 72% maximum binding (OD value 0.6–0.7 at 490 nm). A minimum of 8–16 ng (1.07×10^{-10} M) of antibody was required to bind purified HTNV strains 76–118 and 84Fli. Additionally, as expected, AH100 did not bind SEOV (Fig. 4).

Fine Epitope Mapping of AH100IgG

To define the antigenic determinant on HTNV G1 recognized by AH100IgG, peptide-scanning methods were used. For the initial scanning, 15mer peptides that overlapped by five amino acids, representing the entire G1 protein of HTNV, were synthesized on activated membranes and were probed with affinity purified AH100IgG as described in Methods section. The

TABLE II. Antigenic Analysis of Neutralizing Human Recombinant Antibody AH100IGg by IFA, ELISA and PRNT of Hantaan and Seoul Viruses

Virus	Host	Isolation	Assays		
			IFA[a]	ELISA (OD_{490})[b]	PRNT (%)[c]
HTNV					
76–118	A. agrarius	Korea	++++	0.78	95
A9	A. agrarius	Jiangsu.C	++++	0.63	89
84-Fli	Human	Shanxi.C	++++	0.89	95
Chen	Human	Anhui.C	++++	0.81	92
H5	Human	Helong.C	++++	0.78	92
Luxu	Human	Shandong.C	+++	0.56	NT
B569	Human	Shandong.C	+++	0.62	NT
SEOV					
HR-80-39	R. norvegicus	Korea	—	0.03	—
SR-11	Rat	Japan	—	0.02	—
L99	R. rosea	Jiangxi.C	—	0.01	—
R22	R. norvegicus	Henan.C	—	0.02	—
HB55	Human	Henan.C	—	0.03	NT
DOBV			—	NT	NT
PUUV			—	NT	NT

[a]The AH100IgG antibody tested for all above assays was expressed in High 5 insect cells and the supernatants were diluted 1:2. HTNV and SEOV antigen slides for IFA were prepared as described in methods. DOBV antigen slides were kindly provided by Dr. T. Avsic-Zupanc. PUUV antigen slides were purchased from Progen, Germany. ++++, +++ correspond to approximately 100%, or 75% (respectively) fluorescent cells per field of assay.
[b]Capture ELISAs were performed as described in Materials and Methods.
[c]Plaque Reduction Neutralization Test (PRNT) were performed in 24-well plates as described in Materials and Methods. The value shown represents the % neutralization of approximately 35 plaques per well. NT, not tested.

antibody was highly reactive to the peptide spot corresponding to the amino acid sequence QRKVILTLVIGQC located at amino acids 438–452 of the G1 protein (data not shown). To identify the precise binding site, 15mer peptides, which overlapped by only one amino acid, covering the immunoreactive area were synthesized and probed as before. In this assay, the highest antibody binding was to the peptide GQRKVILTKLVIGQ (amino acid 437–451) (Fig. 5). To further define amino acids crucial to the epitope, the sequence GQRKVILTKLVIGQC was subjected to a mutational analysis by successively replacing each amino acid in the 15mer pep-tide with glycine or alanine. Probing of the resulting peptides with AH100IgG, indicated that the replacement at any position of the sequence LTKTLVIGQ (amino acid 443–451) led to a substantial decrease in reactivity (Fig. 6). Glycine or alanine replacements at other positions (Gln 438, Arg 439, Lys 440, Val 441, and Cys 451) in the peptide obviously decreased the affinity of the antibody binding, as indicated by the intensity of the chemiluminescence signal (Fig. 6). Ile442 seems

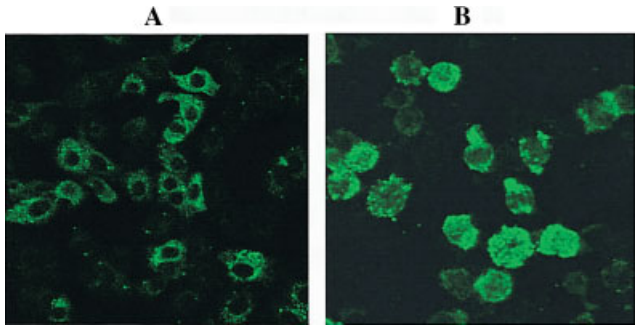

Fig. 2. Immunofluorescence assay of recombinant human antibody AH100IgG with HTNV-infected Vero-E6 cells (**A**) or with HTN M fragment specific recombinant baculovirus infected SF9 cells. (**B**) FITC conjugated anti-human IgG Fc antibodies were used for the detection. Cell culture supernatants of SF9 cells infected with recombinant baculoviruses expressing human IgG antibodies to G1 and N were incubated with the slides at 37°C for 30 min. Bound antibodies were detected with FITC conjugated anti-human human Fc antibodies.

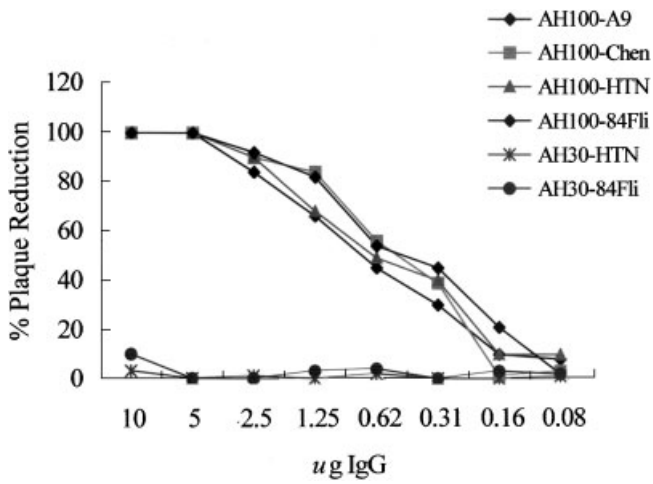

Fig. 3. Plaque-reduction neutralization assay of baculovirus/insect cell-expressed recombinant human antibodies to HTNV. Serial dilutions of the G1 and N-specific human antibodies AH100IgG and AH30 were assayed with HTNV strains A9, Chen, 76–118(HTN), 84-Fli.

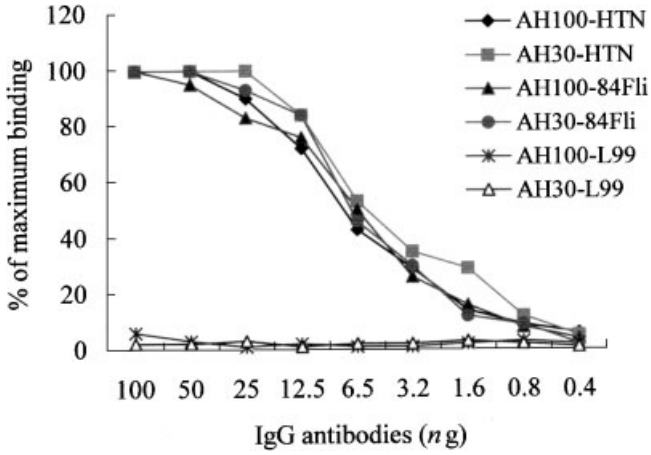

Fig. 4. Binding of recombinant human antibodies AH100IgG (G1-specific) and AH30IgG (N-specific) to sucrose gradient-purified HTNV, strains 76–118 and 84Fli, and SEOV, strain L99 as measured by ELISA. Values are expressed as % of maximum binding of OD value at 490 nm.

uninvolved in antibody binding, as indicated by no loss of reactivity of peptide 6 as compared to that the non-mutated peptide (Fig. 6). These results suggest that the neutralizing antibody AH100 recognized a linear epitope in the synthesized peptide spots assay.

DISCUSSION

Phage display and recombinant antibody technologies have been used successfully to generate human antibodies to a wide range of viral pathogens [Burton and Barbas, 1994; Parren et al., 2000; Sawyer, 2000]. Generally, antibodies are thought to have evolved to recognize and eliminate foreign pathogens by targeting surface molecules. For some viral diseases, the therapeutic use of human-derived antibodies offers an alternative to antiviral drug treatment. In addition, human antibodies provide important tools to explore the molecular basis and mechanisms of viral infection and antiviral immune protection. In this study, we used phage display technology and RNA extracted from the blood of a convalescent HFRS patient to generate human Fab to HTNV, the most common cause of HFRS. Because it is known that neutralizing antibodies can be generated

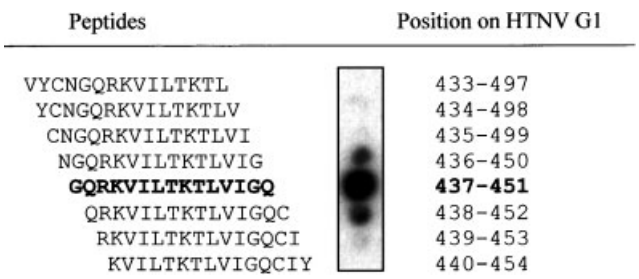

Fig. 5. Fine epitope mapping of the neutralizing human recombinant antibody AH100IgG. The epitope region (aa 437–452) was selected for fine mapping based on results obtained by screening overlapping peptides corresponding to the entire G1 protein of HTNV (see Materials and Methods).

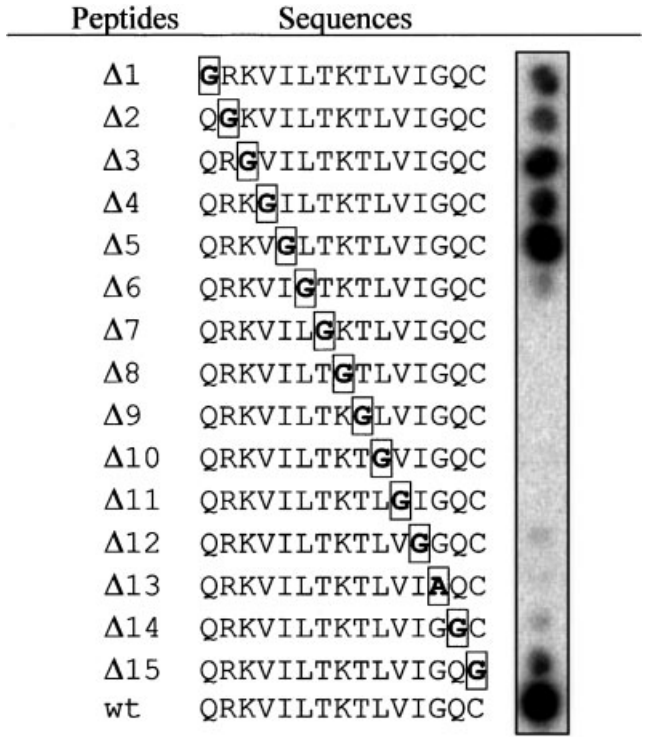

Fig. 6. Mutational analysis of aa 437–452. Fifteen peptides were synthesized; each amino acid of the wild-type (wt) peptide was replaced by Glycine or Alanine, as indicated by boxed bold lettering. The resulting mutated peptides were incubated with the human IgG antibody IgG100 and analyzed as the methods for Figure 5.

to both of the viral glycoproteins, G1 and G2, and that neutralizing antibodies are associated with protective immunity, we were most interested in obtaining antibodies reactive with G1 or G2. Consequently, we selected our library by panning both on complete virus, and on envelope protein antigens that were captured with mouse Mabs to G1 and G2. Despite these procedures, we selected several Fabs reactive with N, as well as several reactive with the envelope protein antigens. Nucleotide sequence analysis of the CDR3 coding regions of 10 selected Fabs revealed only two distinctly different predicted amino acid sequences, one of which corresponded to N-reactive Fabs and the other with Fabs reactive with the envelope glycoproteins. Therefore, we surmise that all of the Fabs were likely derived from two parent clones. By radioimmune precipitation, we determined that all of the envelope-protein reactive Fabs recognized G1. Of these, we selected two with neutralizing activity and one of the N-reactive Fabs for conversion to complete IgG. We next constructed recombinant baculoviruses expressing complete IgGs and characterized the expressed antibodies with respect to binding and neutralization of HTNV and other viruses causing HFRS.

Four hantaviruses are known to cause HFRS of varying clinical severity, ranging from asymptomatic infection to fulminate hemorrhagic shock and death. HTNV and DOBV cause the most severe forms of HFRS with the highest mortality rates. These viruses are both

carried by *Apodemus* field mice. SEOV, carried by rats, and PUUV, carried by *Clethrionomys* bank voles, cause serious disease, but less frequent fatalities. The molecular and pathogenic mechanisms responsible for the variation in clinical severity of HFRS are poorly understood. Likewise, the human immune responses, including the exact targets of neutralizing antibodies, are not well defined. We were able to identify one target of a human immune response that might be important for protective immunity. The source of the human G1-specific antibody that we studied was an HFRS patient from Anhui province, in southern China. This is an endemic HFRS region with severe disease typical of HTNV infection. ELISA analysis of the patient sera before use in our studies revealed significant neutralizing activity to HTNV (1:400) by PRNT, and high titers to N (1:1,280), G1(1:512) and G2 (1:256) (data not shown). Later IPs also showed the patient has antibodies to both G1 and G2 (Fig. 1B). Consequently, it is not known why we did not obtain Fabs reactive with G2. Because this library was selected from only one patient sample, it is not likely to be representative of the immune responses of HFRS patients in general.

Previous studies demonstrated that antibodies to the G1 proteins of Old World hantaviruses are more type-specific than those to G2 are, i.e., monoclonal antibodies to G1 react only with homologous virus, but those to G2 can cross-react with other hantaviruses [Arikawa et al., 1989; Lundkvist et al., 1991, 1993; Avsic-Zupanc et al., 1995]. Consistent with these findings, our patient-derived human recombinant antibody, AH100, recognized HTNV but not SEOV, PUUV, or DOBV in IFA. Similarly, this antibody recognized and neutralized HTNV but not SEOV when examined by ELISA and PRNT. These data may have implications on the design of an immunotherapeutic approach to HFRS in regions where more than one hantavirus causes HFRS. It might be necessary, for example, to use a cocktail of antibodies to treat HFRS when the infecting virus has not yet been identified.

To gain a better understanding of the reactivity of the G1-specific antibody AH100, we mapped its binding site by reacting it with a series of overlapping peptides representing the G1 protein of HTNV. Subsequently, we carried out sequential single amino acid mutational analysis to identify specific amino acids involved in site recognition. The results of our study indicate that AH100 recognizes a core portion of the G1 epitope with a sequence of LTKTLVIGQ (amino acid 443–451 of HTNV G1). The affinity of the antibody binding is increased by the surrounding amino acids (Gln 438, Arg 439, Lys 440, Val 441, and Cys 451).

The epitope that we defined for AH100 is located in the C-terminal hydrophilic region of G1, which may be exposed on the surface of HTNV. This epitope is close to a site (aa 415) where a neutralizing mouse Mab (16D2) was mapped by using antibody-escape mutants [Wang et al., 1993]. It is also near a site identified in an attenuated HTNV mutant (mu11E10, aa 515), which was postulated to be related to virulence in mice [Ebihara et al., 2000]. This defined epitope is also in line with the region of DNA segment from aa 399–565 that induced the neutralizing antibody responses to SNV [Bharadwaj et al., 1999]. Non-neutralization epitopes have been mapped to the N-terminal third of the G1 protein of hantaan virus [Wang et al., 1993], however, the evidences described above indicated that the C-terminal third of G1 protein constitute one of major neutralization epitopes of hantaviruses and may contribute to bind the virus receptor on infected cells and related to virus virulence.

Despite our finding that AH100 IgG is HTNV-specific, we found that the core epitope was mostly conserved among all four HFRS-causing hantaviruses. One possible explanation for this is a differing availability of the site on the virion surface because of protein conformation. We found that there is a conserved N-linked glycosylation sites Asn-X-Ser nearby the epitope (15 aa downstream of the epitope); different sugars may contribute to the conformation of the antibody-binding domain. Alternatively, differences in amino acids adjacent to the epitope may influence the ability of the antibody to bind to the core epitope. Comparing the HTNV-derived peptide QRKVILTKTLVIGQC to the allied peptides derived from SEOV, DOBV, and PUUV (Table III), the adjacent amino acids to the conserved

TABLE III. Comparison of the Predicted Amino Acid Sequences for Various Hantaviruses at the Defined Binding Site of the HTNV G1-Specific Antibody AH100IgG

Virus strains	Peptide sequences[a]	Accession number
HTN		
76-118, HoJo, A9, HV114	VVYCN^{437}GQ**R**K**V**I<u>LTKTLVIGQ</u>^{451}CIYTI	D00376, M14627, U00150, L08753
SEO		
HR80-39, SR-11, L99HB55	IVYCN^{435}GQ**K**K**T**I<u>LTKTLVIGQ</u>^{451}CIYTI	AB027561, M34882, AF035833, AF035833
DOB		
Dobrava	VIYCN^{437}GQ**K**K**T**I<u>LTKTLVIGQ</u>^{451}CIYSV	L33685
PUU		
Sotkama	IVYCN^{447}GM**K**K**V**I<u>LTKTLVIGQ</u>^{461}CIYSV	X61034
P360	IVYCN^{447}GLKKVI<u>LTKTLVIGQ</u>^{461}CIYTP	L087

[a]The amino acid sequences are numbered based on the amino terminal amino acid sequence deduced for G1 and are included with the numbers that represent the amino acid positions of each peptide. The core amino acid sequences of HTNV G1 defined as the binding epitope for AH100 and the identical sequences found in deduced amino acid sequences of SEOV, DOBV and PUUV G1 are underlined. The different adjacent amino acids to the conserved core epitope LTKTLVIGQ are in bold.

core epitope LTKTLVIGQ revealed differences between HTNV (Arg 439) and SEOV, DOBV (Lys 437) as well as PUUV (Lys 449) at the allied position. The binding signal of AH100IgG to the peptide was decreased significantly by mutating the Arg 439 to Glycine (Table III). A third possibility is that the linear core epitope we defined is only a part of a conformational epitope. In support of this theory, we found that AH100 reacted with intact G1 by immune precipitation, but not by Western blot (data not shown). Similar findings; i.e., that a portion of a conformational epitope can be recognized by an antibody in a highly sensitive assay involving short linear peptides, have been reported previously [Roivaine et al., 1991; Heiskanen et al., 1999]. Furthermore, crystallographic studies on antigen-antibody complexes showed that antibodies may directly interact with 14–22 amino acids and that 2–5 continuous linear peptides constitute one conformational antibody binding domain on an antigen [Davis and Padlan, 1990].

In summary, we generated a type-specific, neutralizing human IgG to HTNV G1 by using phage display and recombinant antibody technology. This antibody and other similar antibodies derived from HFRS patients may prove useful for immunotherapy or for studies aimed at defining the human immune responses to hantaviruses.

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