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Bacterial Expression of Neutralizing Mouse Monoclonal Antibody

MIFANG LIANG, YONG-KYU CHU, and CONNIE SCHMALJOHN[1]

Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21702-5011

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We amplified by polymerase chain reaction the heavy and light chain antibody genes of two mouse hybridomas secreting neutralizing monoclonal antibodies (MAbs) to the G1 or G2 envelope proteins of Hantaan virus, cloned them into the phagemid vector pComb3, and expressed them in bacteria to yield Fab fragments. Expressed Fab fragments had the same antigenic specificities for Hantaan and Seoul viruses as the complete parent MAbs and were able to neutralize Hantaan virus in plaque-reduction neutralization assays. The authentic MAb to G2 (HCO2) could passively protect hamsters from challenge with Hantaan virus when neutralizing antibody titers of at least 1:10 were detected in the animals' sera just prior to challenge. In contrast, although 1:10 neutralization titers were also detected in hamsters receiving passively transferred, *Escherichia coli*-expressed HCO2 Fab, these animals were not protected from infection with Hantaan virus. Similarly, passive transfer of the HCO2 MAb on Days 1 through 4 after infection prevented antigen deposition in hamster lungs and kidneys but passive transfer of the recombinant HCO2 Fab did not. The results suggest that although neutralization by IgG antibodies correlates with protection in hamsters, the same may not be true for neutralizing Fab fragments.

INTRODUCTION

Viruses in the *Hantavirus* genus, family Bunyaviridae, are known to cause two serious and often fatal diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome. Approximately 200,000 hospitalized cases of HFRS occur annually, about half of them in China. Hantaviruses are carried by specific rodent hosts and are transmitted as infectious aerosols of urine, feces, or saliva (reviewed in Lee and Dalrymple, 1989). At least four hantaviruses cause HFRS: Hantaan (HTN) virus, carried by *Apodemus agrarius* (striped field mice); Seoul (SEO) virus, carried by *Rattus novegicus* (domestic rats); Dobrava virus carried by *Apodemus flavicolis* (yellow-necked mice); and Puumala virus, carried by *Clethrionomys glariolus* (bank voles) (Lee *et al.,* 1978, 1982; Brummer-Korvenkontio *et al.,* 1980; Avsic-Zupanc *et al.,* 1992; Taller *et al.,* 1993). Several hantavirus vaccines are currently undergoing clinical trials, but no vaccine has yet been proven effective for preventing HFRS. Therapeutic measures are generally limited to supportive care, although the drug ribavirin improved disease outcome in a trial performed in China (Huggins *et al.,* 1991).

Immunotherapy for treatment or prophylaxis of hantaviral infections of humans has not been widely studied. HFRS patients remain viremic during the acute phase of disease as evidenced by the isolation of viruses from blood collected during this time (Schmaljohn *et al.,* 1988; Antoniades *et al.,* 1987; Avsic-Zupanc *et al.,* 1994; Lee and Dalrymple, 1989). Intervention with therapeutic neutralizing human monoclonal antibodies (MAbs) might, therefore, prove useful for shortening the viremic period of HFRS. Neutralizing antibodies were also found to passively protect animals from challenge with HTN or SEO viruses (Antoniadis *et al.,* 1989; Schmaljohn *et al.,* 1990; Zhang *et al.,* 1989; Arikawa *et al.,* 1992); thus, neutralizing human MAbs might prove useful for postexposure prophylaxis.

Methods have recently been developed for generating combinatorial human antibody libraries by expressing antibody genes obtained from immune individuals. Phage display of antibody fragments has proven to be a useful means for selecting antibodies of the desired specificity and has resulted in the development of murine or human MAbs to a number of viruses including influenza virus, human immunodeficiency virus, rabies virus, respiratory syncitial virus, and hepatitis B virus (Caton and Koprowski, 1990; Burton *et al.,* 1991; Cheung *et al.,* 1992; Barbas *et al.,* 1992; Zebedee *et al.,* 1992). As a first step toward developing human MAbs as a therapeutic reagent for treatment of HFRS, we cloned and expressed in bacteria the heavy and light chain antibody genes from two mouse hybridomas secreting HTN virus G1- or G2-specific MAbs and evaluated the resultant Fab fragments for their ability to recognize viral antigens and to neutralize HTN virus in cell culture. We also compared the ability of the anti-G2 Fab to that of the parent MAb for the ability to passively protect hamsters from challenge with virulent HTN virus.

[1] To whom reprint requests should be addressed.

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MATERIALS AND METHODS

Virus and cells

HTN (76-118) and SEO (SR-11) viruses were propagated in Vero–E6 cells (ATCC C1008, CRL1586), concentrated by precipitation with polyethylene glycol, and purified by sucrose gradient sedimentation as previously described (Schmaljohn *et al.,* 1983). The mouse hybridoma cell lines secreting neutralizing MAb to G1 (2D5) and G2 (HCO2) were described previously (Arikawa *et al.,* 1989).

Cloning of heavy and light chain antibody genes

Total cellular RNA was isolated from pelleted hybridoma cells with Trizol reagent (Gibco BRL, Gaithersburg, MD). cDNA was synthesized by using oligo(dT) primers and reverse transcriptase (Boehringer Mannheim cDNA Synthesis kit, Indianapolis, IN) and was amplified by polymerase chain reaction (PCR) with specific heavy and light chain primers of murine IgG. Forward primers used were HC1 (5′-AGGTCCAGCTGCTCGAGTCTGG-3′) and Vk (5′-CCAGATGTGAGCTCGTGATGACCCAGTCTCCA-3′), and reverse primers were IgG1 (5′-AGGCTTACTAGTACA-ATCCCTGGGCACAAT-3′), IgG2a/2b (5′-GATATCACT-AGTGGGCCCGCTGGGCTC-3′), and Ck2 (5′-GCGCCG-TCTAGAATTAACACTCATTCCTGTTGAA-3′) (Kang *et al.,* 1991). Thirty-five PCR cycles were performed, each consisting of 94° for 1 min, 52° for 1 min, and 72° for 2 min. The resultant PCR products were gel-purified in agarose and extracted using Spin-X columns (Costar, Broadway/Cambridge, MA). The heavy chain Fd and light chain genes were then successively cloned into the phagemid expression vector pComb3 (Barbas *et al.,* 1991) using *Xho*I/*Spe*I and *Sac*I/*Xba*I sites, respectively.

Escherichia coli expression and purification of soluble Fab fragments

The ligated pComb3 vector with heavy chain Fd and light chain PCR DNAs was electroporated into competent XLI-Blue *E. coli* cells (Stratagene, La Jolla, CA) by using a BRL Cell-Porator Electroporation system, with settings of 400 to 420 voltage charge, and a final delivery electric pulse of 2.5 V (BRL Life Technologies, Inc., Gaithersburg, MD). After transformation, cells were streaked on LB/ampicillin plates and the clones with both inserts were selected by colony screening by methods similar to those published previously (Barbas *et al.,* 1991). Briefly, bacterial colonies were induced with isopropyl B-D-thiogalactopyrano-side (IPTG), and colonies were transferred to nitrocellulose and treated with chloroform. The filters were blocked with phosphate-buffered saline, pH 7.4, containing 3% bovine serum albumin (PBS–BSA). Filters were incubated with purified HTN virus antigen diluted in PBS–BSA, washed in PBS containing 0.1% Tween-20, and incubated with MAbs 11E10 and 6D4 (Arikawa *et al.,* 1989) to the G1 and G2 proteins of HTN virus which were conjugated with horseradish peroxidase (HRP) by a method described previously (Harlow and Lane, 1988). After selection, the phagemids were reisolated, digested with *Spe*I and *Nhe*I to remove the gene III fragment, and religated to yield a phagemid capable of expressing soluble Fab fragments. These clones, designated 2D5 Fab and HCO2 Fab, were grown in super broth medium [30 g tryptone, 20 g yeast extract, 10 g MOPS buffer per liter, pH 7.0 (containing 50 μg ampicillin/ml)] until an OD_{600} of 0.2 to 0.4 was achieved. Then 1 m*M* IPTG was added and the culture was grown overnight at 30° or at room temperature. Cells were pelleted by centrifugation and lysed by three cycles of freezing at −70° and thawing at room temperature. Cell lysates were clarified by centrifugation at 12,000 to 14,000 rpm in a microcentrifuge at 4° for 15 min and the resultant supernatants were filtered through 0.45-μm filters and applied to an affinity column, which was prepared by covalently coupling purified anti-mouse IgG Fab antibodies (Sigma, St. Louis, MO) to protein G–Sepharose 4B (Sigma) with dimethylpimelimidate, as described previously (Harlow and Lane, 1988). The Fab proteins were eluted with a buffer consisting of 1.0 *M* glycogen–HCl (pH 2.7) and were neutralized by adding 1 *M* Tris–HCl, pH 9.0. The eluted fractions were concentrated by using Centricon-10 filters (Amicon, Beverly, MA) and protein concentrations were determined with the BCA protein assay kit according to the manufacturer's directions (Pierce, Rockford, IL).

Preparation and purification of IgG and Fab fragments

Complete IgG molecules of the neutralizing MAbs 2D5 and HCO2, and a control nonneutralizing MAb to the nucleocapsid protein of HTN virus, BDO1 (Ruo *et al.,* 1991), were purified from mouse ascitic fluids by using an IgG purification kit, MAbTrap G (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Fab fragments were prepared by using the Immuno Pure Fab Preparation kit (Pierce, Rockford, IL). Briefly, the purified IgG described above was digested with agarose-immobilized papain at 37° for 5 hr, the crude digest was applied to an immobilized protein A column, and the Fab fragments were eluted and concentrated. Twenty microliters each of the purified IgG, papain-derived, and recombinant Fabs (0.9–1.0 mg/ml) were examined by SDS–polyacrylamide gel electrophoresis (PAGE) in 12.5% acrylamide/DATD gels as previously described (Schmaljohn *et al.,* 1983). Gels were fixed and then stained with 0.2% Coomasie brilliant blue.

Immunoprecipitation of Hantaan virus with the recombinant Fabs

Confluent monolayers of Vero–E6 cells in 25-cm^2 flasks were infected with 1 ml of HTN virus (strain 76-

118, 1.9×10^6 PFU). At 24 hr postinfection, the cell culture medium was removed and replaced with cysteine- and methionine-free Eagle's minimal essential medium, Earles' salts, containing 2% FBS, and incubated for 1 hr at 37°. Cells were radiolabeled with 200 μCi of [^{35}S]Promix (Amersham, UK) per flask for 4 hr, after which they were lysed on ice with 1 ml of a buffer composed of 10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA, 0.5 *M* NaCl, 0.25 mg/ml each aprotinin and α-2-macroglobulin, and 4% Zwittergent 3-14 (Calbiochem–Behring, La Jolla, CA). Nuclei were removed by centrifugation. Radiolabeled HTN virus proteins were immune-precipitated by mixing 100 μl of cell lysate with 100 μl of the clarified bacterial lysates containing recombinant Fabs. The mixture was incubated at 4° overnight after which anti-mouse IgG Fab antibodies bound to protein G–Sepharose 4B (Sigma, St. Louis, MO) were added and incubated with shaking at 4° for 2 hr (Harlow and Lane, 1988). The immune-precipitated proteins were washed three times with lysis buffer with 1% Zwittergent and once with 0.1 *M* Tris–HCl, pH 8.0, 1 m*M* EDTA. SDS–PAGE was as described above.

Plaque-reduction neutralization test (PRNT)

PRNT assays were performed on Vero–E6 cell monolayers in six-well cell culture plates with HTN and SEO viruses using methods similar to those published previously (Schmaljohn *et al.,* 1988). Briefly, the purified antibodies, including IgG, papain-derived Fabs, and recombinant Fabs, were standardized to 50 μg per milliliter and were serially diluted in twofold or fourfold increments. Sera from hamsters challenged with HTN virus were initially diluted 1:10 and then in twofold increments. The serum dilutions were incubated with 100 PFU of HTN or SEO viruses at 37° for 1 to 2 hr. Virus–antibody mixtures were applied to cell monolayers in six-well plates and incubated at 37° for 1 hr in a 5% CO_2 incubator and then overlaid with EMEM containing 0.6% agarose (Seakem ME, FMC Corp.). After incubation at 37° for 8 days, a second overlay identical to the first, except for the addition of neutral red (final concentration, 0.167 mg/ml), was added to the plates. The neutralizing antibody titer was calculated as the reciprocal of the highest dilution resulting in an 80% reduction of plaques in controls.

ELISA

Sucrose gradient-purified HTN or SEO virions (Schmaljohn *et al.,* 1983) were diluted in PBS, pH 7.4, and 100 μl was added to each well of flat-bottomed 96-well microtiter plates (Corning, NY) and incubated at 4° overnight. The plates were washed three times with PBS containing 0.05% Tween-20, and 100 μl of twofold dilutions of the purified, recombinant Fabs (starting at 20 μg/ml protein concentration) was added to the wells. The plates were incubated at 37° for 1.5 hr. The plates were washed as described above and 100 μl of a 1:2000 dilution of HRP-conjugated anti-mouse Fab (Sigma, St. Louis, MO) was added and incubated at 37° for 1 hr. After washing, 100 μl of ABTS peroxidase substrate (Kirkegard & Perry Laboratories, Gaithersburg, MD) was added and the plates were incubated for 20 min at room temperature. Relative binding avidity was defined as the amount of purified Fabs (μg Fab protein) required to yield an absorbance of 0.4–0.5 at 405 nm in a Bio-Lab ELISA reader.

Nucleotide sequence analysis

Plasmid DNA was prepared by using the Qiagen miniprep kit (Qiagen, Charsworth, CA). Nucleotide sequences of the variable regions of the heavy and light chain genes were determined by the dideoxy chain termination method by using a Sequenase version 2.0 sequencing kit (U.S. Biochemical, Gaithersburg, MD) according to the manufacturer's directions. A primer (5′-CTAACTAGCTAGTCGCC-3′) corresponding to pComb3 sequences was used to determine the nucleotide sequence of the variable region of the light chain. Because vector sequences near the 5′ terminus of the heavy chain inserts existed in multiple copies in pComb 3, it was necessary to subclone the heavy chain gene fragment into a different plasmid for sequence analysis. Consequently, the heavy chain fragment was cloned into PCRII vector (Invitrogen Corp., San Diego, CA) and the sequence was determined by using the M13 reverse primer (Invitrogen). At least three independently selected clones were used to determine each sequence. The variable region sequences of light and heavy chains were analyzed by using MacVector Sequence Analysis software (International Biotechnologies, Inc.) on a Macintosh computer. The complementarity determining regions of 2D5 and HCO2 antibodies were located by comparing sequences to those reported previously (Padlan, 1994).

Passive protection of hamsters

Two different protection experiments were conducted. In the first study, approximately 180 to 200 μg of purified IgG from the G2-specific neutralizing MAb HCO2 or from the nonneutralizing N-specific MAb BDO1 or recombinant HCO2 Fab fragments were each passively administered by intramuscular injection (im) to four female 8- to 10-week-old outbred Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, MA). Control hamsters received PBS without antibodies. Five hours later, the hamsters were challenged by im injection of 10^3 PFU of HTN virus. Hamsters were bled the day before challenge and 28 days after challenge. Blood samples were examined for the presence of virus-specific antibodies by using a direct immunofluorescent antibody assay (IFA) and HTN virus-infected Vero–E6 cells on spot slides (Arikawa *et al.,* 1989) and by PRNT. Animals

were killed on Day 28 postinfection, and the lung and kidney tissues were harvested and examined for the presence of viral antigen by IFA as described previously (Chu *et al.,* 1995).

In the second study, hamsters were first infected with 10^3 PFU of HTN virus by im injection, and the purified antibodies described above were passively administered on Days 1, 2, 3, and 4 after infection (200 μg/day). The hamsters were bled from the orbital venous sinus on Days 7, 14, and 28 after administration of antibodies. In addition to IFA detection of viral antigen in tissues, nested RT–PCR was used to monitor viremia and virus distribution in tissues as described elsewhere (Chu *et al.,* 1995). Briefly, total cellular RNA was extracted from 100 μl of blood or 10% lung, or kidney suspensions with Trizol reagent (Gibco BRL), and HTN-specific primers were used for RT–PCR. The outer primers used were 5′-GGACCTGGTGCCAGTTGTGAAGC-3′ and 5′-ACCTCACAAACCATTGAACC-3′ (1190–1680 with respect to HTN virus sequence), and the inner primers were 5′-GTACTGATTTTAGCCTATTCTC-3′ and 5′-TGCAACGGGCAGAGGAAAGT-3′ (1269–1625 with respect to HTN sequences). PCR was performed for 35 cycles for 30 sec at 94°, 30 sec at 50°, and 2 min at 72°.

RESULTS

Expression and characterization of Fab fragments

We PCR-amplified and cloned into pComb3 the heavy chain Fd region and complete light chain antibody genes from two mouse hybridomas secreting neutralizing antibodies to HTN virus G2 (clone HCO2) or G1 (clone 2D5). Expression of both genes was controlled by the *E. coli* lac promoter/operator (Barbas *et al.,* 1991). The heavy chain was expressed as a fusion protein with phage coat protein III (cpIII), and the light chain was expressed independently. A pelB leader sequence preceding both genes directed the expression products to the bacterial periplasmic space where assembly into Fab fragments occurred. Because of the cpIII portion of the heavy chain fusion protein, the Fabs remained tethered to the inner bacterial membrane. Because the Fab genes were extracted from the hybridoma cells, it was not necessary to use phage display techniques or panning on specific antigen to select for recombinant clones. Instead, to identify specific bacterial clones expressing the Fabs of interest, we performed colony-screening assays on the bacteria transformed with the pComb3 vectors by using gradient-purified HTN virus antigen as described under Materials and Methods. Most, but not all, colonies expressed antibodies capable of recognizing HTN virus (data not shown).

After selecting clones expressing HTN virus-specific Fabs, we recovered phagemid DNA and removed the gene III fragment to allow production of soluble Fabs, which were secreted into the periplasmic space of the bacteria. Total cell lysates were prepared and ELISA was used to confirm that the HCO2 and 2D5 Fabs reacted with both anti-mouse Fab antibody and purified HTN virus (not shown).

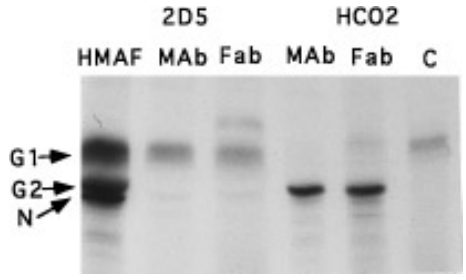

FIG. 1. Polyacrylamide gel electrophoresis of radiolabeled HTN virus proteins immune-precipitated with HTN virus-specific antibodies. Lanes from left to right show products precipitated with hyperimmune mouse ascitic fluid (polyclonal) to HTN virus (HMAF), mouse ascitic fluid containing 2D5 MAb, bacterial lysates containing recombinant 2D5 Fab, mouse ascitic fluid containing HCO2 MAb, bacterial lysate containing recombinant HCO2 Fab, and a control bacterial lysate containing only the phagemid pComb 3 (C). The positions of HTN virus G1, G2, and N are indicated to the left of the autoradiogram.

To determine if the Fabs retained the ability to recognize HTN virus proteins, the clarified supernatants from bacterial lysates containing Fab fragments were used to immune-precipitate radiolabeled HTN virus proteins. The HCO2 and 2D5 Fabs were able to recognize and precipitate HTN virus G2 and G1, respectively (Fig. 1).

Sequence analysis of the heavy and light chain antibody genes

To examine the diversity in the heavy and light chain antibody genes of the HCO2 and 2D5 Fabs, the nucleotide sequences encoding the variable domains of heavy and light chains from 2D5 and HCO2 IgG were determined and compared. These sequences were aligned to those in GenBank by using the Wisconsin Sequence Analysis Package software. Such alignment indicated that the sequences belonged to the mouse immunoglobulin family IgG λ chain and κ chain, but were not identical to any other sequences deposited in GenBank. The nucleotide sequences determined for the HCO2 and 2D5 clones were deposited in GenBank under Accession Nos. L46808, L46809, L46813, and L46814. Alignment of the deduced amino acid sequences of the 2D5 and HCO2 variable regions revealed framework regions typical of other mouse immunoglobulin frameworks (Padlan, 1994) that were highly conserved between the HCO2 and 2D5 heavy chains (88.5% amino acid sequence identity) and relatively conserved between the HCO2 and 2D5 light chains (65% amino acid sequence identity). The hypervariable, complementarity determining regions (CDR1–CDR3) of 2D5 and HCO2, in contrast, had little homology (Fig. 2).

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Heavy chain
Clone            FR1              CDR1         FR2            CDR2
2D5    LESGPELVKPGASVKISCKTSGYTFT EYPMH WVKESHGKSLEWIG GINPKNGV--TSYNQ
HCO2   •••••••E••••••••••P•••S•• A•N•N •••Q•N•••••••• K•FDPLPNGG•••••

                    FR3                          CDR3        FR4
2D5    KFKGKATLTVDKSSSTAYMELRSLTSEDSAIYYCAR SEDVLYYYHDY WGQGTSVTVSA
HCO2   •••••••••••••••••F••K•••••••SV••••• GITYG•IF-•• •••••TL••••

Light chain
Clone          FR1               CDR1              FR2          CDR2
2D5    ELVMTQSPASLAVSLGQRATISC RASESVDSYGNSFMH WYQQKPGQPPKLLIY LASNLES
HCO2   ••••••••••••SA•V•ETV••T• ••••NIY••LA---- •••••Q•KS•Q••V• NRKT•AE

                    FR3                       CDR3       FR4
2D5    GVPARFSGSGSRTDFTLTIDPVEADDAATYYC QQNNEDPWT FGGGTKLEIK
HCO2   •••S•••••••G•Q•S•K•NSLQPE•FGS••• •HHYAT•L• ••S•••••L•
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FIG. 2. Deduced protein sequences of heavy and light chain V-genes of MAbs 2D5 and HCO2. FR, framework region; CDR, complementarity determining region. Complete sequences are available in GenBank under Accession Nos. HCO2 VH, L46808; 2D5 VH, L46809; HCO2 VL, L46813; 2D5 VL, L46814.

Purification and assay of recombinant Fabs

For subsequent experiments, it was necessary to purify and assay the recombinant Fabs. Consequently, Fab fragments were purified from bacterial lysates, and IgG MAbs were purified from mouse ascitic fluids by affinity chromatography. Fabs were also prepared by papain digestion of MAbs and purified by affinity chromatography. The purity of the various antibodies and antibody fragments was established by gel electrophoresis. Purified IgG yielded two bands with the expected apparent molecular masses of approximately 50 and 23 kDa. The apparent molecular weights of the heavy chain Fd region and the light chain of the papain-derived Fabs were approximately 25 and 23 kDa, respectively. Because both the heavy and the light chain gene expression products were approximately the same size (23 kDa) (i.e., the heavy chain product was slightly smaller than that of papain-derived Fabs), the products were not resolved from one another by this method (Fig. 3).

Antigen-binding specificity of the recombinant Fabs

Previously, we demonstrated that the G2-specific MAb HCO2 reacted with both HTN and SEO virus antigens in ELISA, but that the G1-specific MAb 2D5 reacted only with HTN virus antigen (Arikawa *et al.,* 1989). To determine if the recombinant Fabs retained these specificities, indirect ELISA with purified HTN and SEO virions were performed. Concentrations of purified Fabs from 0.08 to 2.0 μg/ml were tested. As with the authentic MAbs, recombinant HCO2 Fab reacted with both HTN and SEO virus antigens, while 2D5 Fab reacted only with HTN antigen (Fig. 4). Because of valency differences between the MAbs and Fabs (i.e., equal amounts of MAb and Fab had antigen-binding valencies of 2 and 3 per 150 kDa, respectively), we could not directly compare the avidities of the MAbs and Fabs; however, a relative comparison of binding avidity (defined as the amount of antibody required to yield an absorbance of about 0.4–0.5 at OD_{405} with HTN virus antigen) yielded values of 0.02 μg for the HCO2 Fab, 0.04 μg for the HCO2 MAb, 0.16 μg for 2D5 Fab, and 0.31 μg for the 2D5 MAb (Fig. 4).

Neutralization of infectious virus with the recombinant Fabs

PRNT of HTN and SEO viruses were performed with dilutions of purified MAbs, Fabs resulting from papain

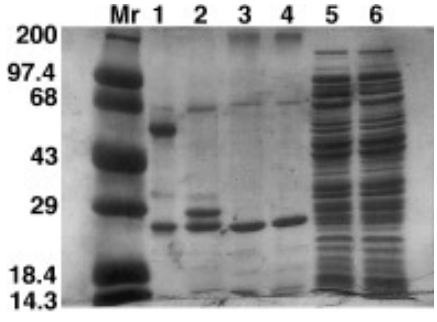

FIG. 3. Coomassie blue-stained polyacrylamide gel. Lane 1, HCO2 IgG purified from mouse ascitic fluid; lane 2, Fabs resulting from papain digestion of HCO2 IgG; lanes 3 and 4, respectively, recombinant HCO2 and 2D5 Fabs purified from bacterial lysates; lanes 5 and 6, unpurified bacterial lysates containing recombinant HCO2 and 2D5, respectively. The positions of stained molecular weight size markers (not shown) are indicated to the left of lane 1.

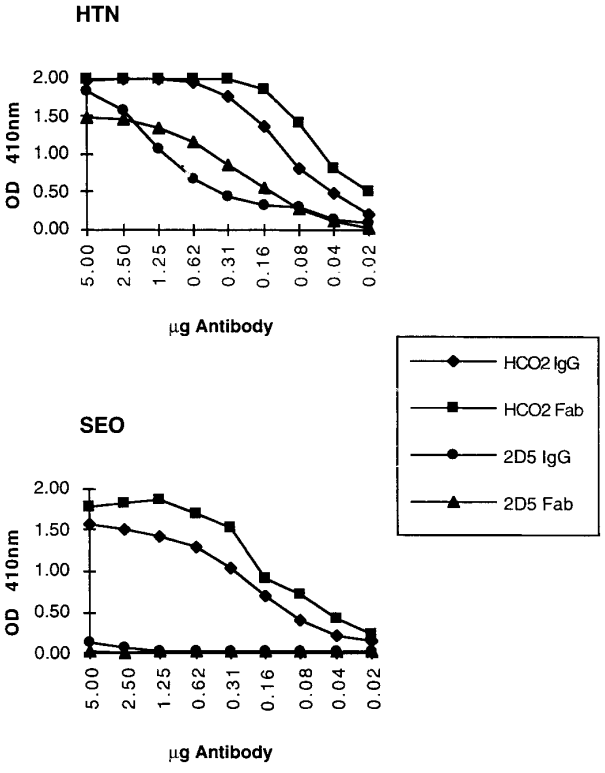

FIG. 4. Specificity of binding of 2D5 and HCO2 Fabs to HTN and SEO virus antigen. ELISA was performed with dilutions of purified, recombinant 2D5 and HCO2 Fabs and sucrose gradient-purified HTN or SEO virions. ELISA results are presented as the amount of antibody needed to achieve OD_{405} nm readings listed on the *Y* axis.

digestion, or recombinant Fabs. HTN virus could be neutralized by all of the antibodies and antibody fragments, but only HCO2 antibodies or antibody fragments were able to neutralize SEO virus. We found that approximately 0.63 μg of the recombinant HCO2 Fab resulted in an 80% reduction in plaques, whereas approximately 2.5 μg of 2D5 Fab was required. Again, without regard to valency differences, these amounts were very similar to those of the authentic MAbs and Fabs required for neutralization (Fig. 5).

Passive protection of hamsters

An animal model that mimics the symptoms of HFRS has not been reported, but a hamster model of protection has been used that is based on the finding that viral antigen can be detected in the lungs of infected hamsters (Schmaljohn *et al.,* 1990). This model was used previously to demonstrate that passively transferred neutralizing MAbs to HTN virus G1 or G2 can protect hamsters from infection with HTN virus (Schmaljohn *et al.,* 1990). In that study, 0.2 ml of nonpurified mouse ascitic fluid containing neutralizing or nonneutralizing MAbs to HTN virus G1 or G2 was administered by the im route to hamsters and the hamsters were subsequently challenged with HTN virus. To more clearly define the amount of the HCO2 IgG MAb required for protection from HTN virus challenge we compared the circulating neutralizing antibody titers achieved 5 hr after transfer of 0.2 ml of unpurified mouse ascitic fluid to those attained after transfer of 50 or 360 μg of IgG purified from the ascitic fluid. We also checked viral antigen deposition in the hamsters' lungs and kidneys 21 days after challenge with HTN virus. We found that transfer of 0.2 ml of unpurified ascitic fluid or 50 μg of purified IgG resulted in neutralizing antibody titers of 10 in three of four hamsters and <10 in one of four hamsters in each group (Table 1). All hamsters with neutralizing titers of at least 10 did not display HTN virus antigen in their lungs after challenge and did not develop detectable postchallenge neutralizing antibodies, indicating that they were protected from infection (Table 1). The two hamsters with neutralizing titers of <10 both had IFA-detectable antigen in their lungs and developed neutralizing antibodies after challenge. Of the four hamsters that received 360 μg of the HCO2 IgG, one animal with a neutralizing antibody titer of 10 displayed very weak fluorescence in lung samples (Table 1). None of these hamsters had detectable postchallenge neutralizing antibodies (Table 1). Thus, a prechallenge neutralization titer of at least 10 was determined to protect hamsters from infection with HTN virus.

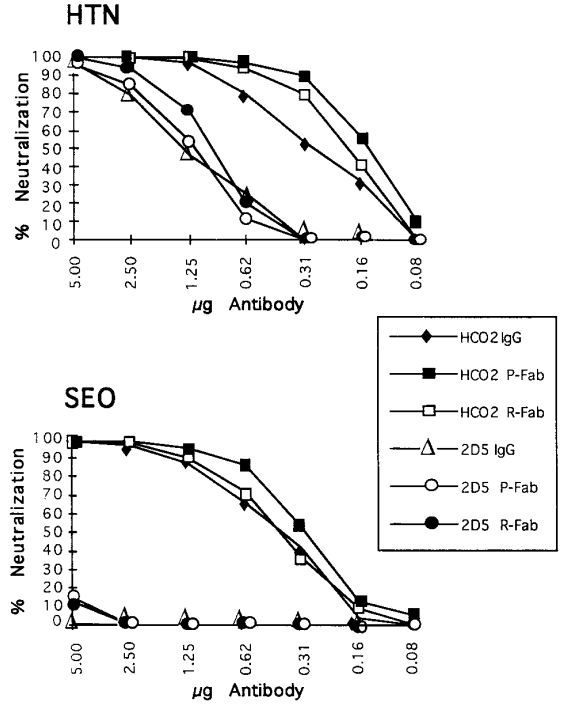

FIG. 5. Plaque-reduction neutralization test (PRNT) with HTN and SEO viruses. After standardization to 50 μg per milliliter, twofold dilutions of purified 2D5 and HCO2 MAbs, Fabs resulting from papain digestion (P-Fab) or recombinant Fabs (R-Fab), were assayed by PRNT. The *X* axis indicates amounts of antibody required to achieve % neutralization (*Y* axis) of approximately 100 plaques.

TABLE 1

Passive Protection of Hamsters with a Neutralizing G2-Specific MAb to HTN Virus

Inoculum[a] and hamster	PRNT antibody[a] Pre	PRNT antibody[a] Post	Antigen[b] in lungs
HCO2 ascitic fluid (0.2 ml)			
1	<10	40	+++
2	10	<10	−
3	10	<10	−
4	10	<10	−
HCO2 IgG (50 μg)			
1	<10	10	+
2	10	<10	−
3	10	<10	−
4	10	<10	−
HCO2 IgG (360 μg)			
1	10	<10	−
2	40	<10	−
3	40	<10	−
4	10	<10	±

[a] Antibody titers were measured by PRNT 5 hr after antibodies were transferred (pre) and 21 days after challenge (post) and are expressed as the reciprocal of the highest dilution neutralizing 80% of plaques in controls.

[b] Viral antigen in lungs and kidneys was monitored by IFA 21 days after challenge, ++++, +++, ++, and + correspond to approximately 100, 75, 50, and 25% fluorescent cells per field of assay. ± indicates very weak fluorescence in only a few cells.

We next compared the ability of authentic HCO2 MAb and recombinant HCO2 Fabs to protect hamsters from challenge. Based on the results presented in Table 1, a dose of 200 μg of HCO2 IgG was selected in order to achieve circulating neutralizing antibody titers of at least 10. An equivalent quantity (approximately 200 μg) of purified, *E. coli*-expressed HCO2 Fab and a control, nonneutralizing antibody were injected into hamsters, and the hamsters were challenged 5 hr later with HTN virus. As before, immediately before challenge, the hamsters were bled and the neutralizing antibody titers to HTN virus were determined. All of the hamsters receiving HCO2 IgG and four of five hamsters receiving HCO2 Fab had neutralizing antibody titers of at least 10 before challenge (Table 2). The hamsters that received a control, nucleocapsid-specific MAb (BDO1) and hamsters that received a mock preparation did not have detectable neutralizing antibodies before challenge. Twenty-one days after challenge, only the hamsters given authentic HCO2 MAb had no PRNT-detectable antibodies to HTN virus and these animals were also the only ones that had no detectable HTN virus antigen in their lungs or kidneys (Table 2). These data indicate that the passively transferred HCO2 Fabs did not prevent infection with HTN virus under the experimental conditions used.

To determine if passive transfer of Fabs could moderate a prior infection with HTN virus, hamsters were first challenged with HTN virus and then were treated with HCO2 MAb, Fab, or a control MAb on Days 1 to 4 after challenge. Viremia was monitored 7 days after challenge by RT–PCR, and HTN-specific antibody was detected by IFA and PRNT 21 days after challenge. The presence of virus in the hamsters' lungs was also checked 21 days after challenge by IFA and by RT–PCR. Only the hamsters that received HCO2 IgG did not display viremia, did not develop antibodies to HTN virus, and had no detectable virus in their lungs (Table 3).

DISCUSSION

Previous studies demonstrated that MAbs 2D5 and HCO2, which respectively react with the G1 or G2 enve-

TABLE 2

Passive Protection of Hamsters with a Neutralizing G2-Specific MAb to HTN Virus

Inoculum[a] and hamster	PRNT antibody[a] Pre	PRNT antibody[a] Post	Antigen[b] Lungs	Antigen[b] Kidneys
HCO2 IgG				
1	40	<10	−	−
2	40	<10	−	−
3	10	<10	−	−
4	40	<10	−	−
HCO2 Fab				
1	10	40	++++	++++
2	10	40	++++	++++
3	10	40	++++	++++
4	<10	160	++++	++++
5	20	40	++++	++++
BDO1 IgG				
1	<10	160	++++	++++
2	<10	40	++++	++++
3	<10	160	++++	++++
4	<10	160	++++	++++
5	<10	40	++++	++++
Mock				
1	<10	160	++++	++++
2	<10	320	++++	++++
3	<10	160	++++	++++
4	<10	320	++++	++++
5	<10	160	++++	++++

Note. The concentrations of antibodies were all 180 to 200 μg, given im.

[a] Antibody titers were determined by PRNT 5 hr after antibodies were transferred (pre) and 21 days after challenge (post) and are expressed as the reciprocal of the highest dilution neutralizing 80% of plaques in controls.

[b] Viral antigen in lungs and kidneys was observed by IFA 21 days after challenge. ++++ indicates fluorescence in 100% of cells examined.

TABLE 3

Postchallenge Viremia, Antibody, and Viral Antigen and RNA in Hamsters Receiving Passively Transferred Antibodies on Days 1, 2, 3, and 4 after Infection with Hantaan Virus

Inoculum and hamster	Viremia	Antibody[a]		[a]RNA or antigen			
				Lungs		Kidneys	
	7 days PI	IFA	PRNT	IFA	PCR	IFA	PCR
HCO2 Fab							
1	+	160	40	++++	+	++++	+
2	+	≥640	40	++++	+	++++	−
3	+	≥640	40	++++	+	++++	+
4	+	≥640	40	++++	+	++++	+
HCO2 IgG							
1	−	<10	<10	−	−	−	−
2	−	10	10	−	−	−	−
3	−	<10	<10	−	−	−	−
4	−	<10	<10	−	−	−	−
BDO1 IgG							
1	+	≥640	160	++++	+	++++	+
2	−	≥640	40	++	+	++	−
3	+	160	40	+	+	+	+
4	+	40	10	−	+	−	−
Mock							
1	−	≥640	40	++++	+	++++	+
2	+	160	40	++++	+	++++	+
3	+	160	40	++++	+	++++	+
4	+	≥640	40	++++	+	++++	+

Note. The concentrations of antibodies were all 180 to 200 μg, given im. For IFA results, ++++, +++, ++, and + correspond to approximately 100, 75, 50, and 25% fluorescent cells per field of assay.

[a] Serum antibody titers and antigen and RNA in lungs and kidneys were measured 21 days after infection.

lope glycoproteins of HTN virus, can neutralize virus *in vitro* and can passively protect hamsters from HTN virus infection (Arikawa *et al.,* 1989; Schmaljohn *et al.,* 1990). The G2 protein of HTN virus is more highly conserved among differing serotypes of hantaviruses than is the G1 protein (Chu *et al.,* 1994), and the HTN G2-specific MAb HCO2 reacts with both HTN and SEO viruses, while the G1-specific MAb 2D5 reacts only with HTN virus. In this study, we examined Fabs expressed from cloned heavy and light chain antibody genes of hybridomas secreting 2D5 and HCO2. We determined that the recombinant proteins had the same antigenic specificities and neutralizing properties for HTN and SEO viruses as the parent MAbs. Because the recombinant Fabs were monovalent, these results suggest that neither cross-linking of viral particles nor cross-linking of envelope glycoproteins on the viral surface was required for antigen binding or neutralization *in vitro.* Although we did not determine the actual mechanism of neutralization by the Fabs, possibilities include prevention of adsorption of virus to host cell receptors, inhibition of fusion of the viral envelope to the host plasma membrane, or alteration of the hantaviral envelope glycoproteins (conformational changes) that perturb entry of virus into host cell and/or its subsequent uncoating (Dimmock, 1995).

To compare the protective or therapeutic properties of the HCO2 Fab to those of the HCO2 MAb, we passively transferred antibodies to hamsters either before or after challenge with HTN virus. Hamsters that received the complete HCO2 MAb before challenge with HTN virus did not develop viremia and did not display viral antigen in their lungs or kidneys, but the hamsters treated similarly with the recombinant Fab became viremic and displayed viral antigen in their organs. Because Fabs are expected to have a shorter half-life than MAbs (4 to 6 days for IgG2b MAb as compared to less than 12 hr for Fabs) (Vieira and Rajewsky, 1988; Spiegelberg and Wiegle, 1965; Sharkey *et al.,* 1991), it is possible that the 5 hr between transfer of the antibodies and challenge was too long for the Fabs, as reflected by the circulating neutralizing antibody titers which were as much as fourfold lower (1:10 compared to 1:40) in hamsters that received Fabs compared to those that received MAbs. However, we do not think that insufficient quantities of Fabs was the only reason for the absence of protection by the Fabs, because our studies indicated that even

1:10 neutralizing antibody titers of the MAbs completely protected most hamsters from challenge.

The results of our passive transfer studies in which MAbs or Fabs were transferred each of 4 days after challenge yielded similar results. That is, only the hamsters that received the HCO2 MAb did not show evidence of HTN virus infection. In these studies, the half-life of the antibody would be less important.

Our finding that neutralization was not necessarily sufficient for protection is similar to results obtained with neutralizing $(Fab')_2$ fragments to the flavivirus yellow fever virus (Schlesinger *et al.,* 1994) and to the arenavirus LCM virus (Baldridge and Buchmeier, 1992). Among the possible explanations for the inability of those molecules and our Fabs to protect from challenge despite their ability to neutralize virus *in vitro* is that in the absence of an Fc region, there is a coincident inability to participate in several humoral and cellular immune functions that are potentially important for virus clearance. For example, neither opsonization nor activation of complement via the classical pathway can occur if the Fc region is not present. Although either or both of these might be involved in clearance of virus from infected hosts, complement is clearly not critical for neutralization of HTN virus *in vitro,* as indicated by results obtained with heat-inactivated sera (Lee and Dalrymple, 1989) and by the results presented in this report. The absence of the Fc region also precludes antibody-dependent cellular cytotoxicity and activation of natural killer cells (Lynch and Sandor, 1990). It is not known if either of these functions is crucial in the development of immunity to HTN virus. Obviously, further studies are needed to determine the mechanism(s) of antibody-mediated protection.

In conclusion, this study demonstrates for the first time the possibility of using cloned antibody genes to generate neutralizing monoclonal antibody fragments to HTN virus. It also provides evidence that *in vitro* neutralization does not neccessarily correlate with *in vivo* protection. Finally, our results suggest that additional studies are needed to determine if Fab fragments could serve as immunotherapeutic reagents for treatment of HTN virus infections.

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