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A humanized murine monoclonal antibody protects mice either before or after challenge with virulent Venezuelan equine encephalomyelitis virus

Ann R. Hunt,[1] Shana Frederickson,[2] Christopher Hinkel,[2] Katherine S. Bowdish[2] and John T. Roehrig[1]

[1]Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, US Department of Health and Human Services, PO Box 2087, Fort Collins, CO 80522, USA

[2]Alexion Antibody Technologies, San Diego, CA 92121, USA

Correspondence
Ann R. Hunt
arh4@cdc.gov

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A humanized monoclonal antibody (mAb) has been developed and its potential to protect from or cure a Venezuelan equine encephalomyelitis virus (VEEV) infection was evaluated. The VEEV-neutralizing, protective murine mAb 3B4C-4 was humanized using combinatorial antibody libraries and phage display technology. Humanized VEEV-binding Fabs were evaluated for virus-neutralizing capacity, then selected Fabs were converted to whole immunoglobulin (Ig) G1, and stable cell lines were generated. The humanized mAb Hy4-26C, designated Hy4 IgG, had virus neutralizing capacity similar to 3B4C-4. Passive antibody protection studies with purified Hy4 IgG were performed in adult Swiss Webster mice. As little as 100 ng of Hy4 IgG protected 90 % of mice challenged with 100 intraperitoneal (i.p.) mean morbidity (MD_{50}) doses of virulent VEEV (Trinidad donkey) 24 h after antibody transfer; also, 500 μg of Hy4 IgG protected 80 % of mice inoculated with 100 intranasal MD_{50} doses of VEEV. Moreover, 10 μg of passive Hy4 IgG protected 70 % of mice from a VEEV challenge dose as great as 10^7 i.p. MD_{50}. Hy4 IgG also protected mice from challenge with another epizootic VEEV variety, 1C (P676). Importantly, therapeutic administration of the humanized mAb to mice already infected with VEEV cured 90 % of mice treated with Hy4 IgG within 1 h of VEEV inoculation and 75 % of mice treated 24 h after virus infection.

INTRODUCTION

Venezuelan equine encephalomyelitis virus (VEEV), an alphavirus in the family *Togaviridae*, is maintained in a natural transmission cycle between mosquitoes and small rodents. It has caused human and equine outbreaks in the Americas for nearly a century (Johnstone & Peters, 1995; Monath & Trent, 1981). Equine epizootics have high mortality (38–83 %) and often lead to human epidemics involving thousands of cases (Groot, 1972; Rivas *et al.*, 1997). Human disease is usually self-limiting with 1–4 % of cases progressing to severe encephalitis (Bronze *et al.*, 2002). VEEV has also caused laboratory-acquired human infections via parenteral or airborne inoculation.

Experimental VEEV vaccines have been developed primarily for protection of laboratory workers and military troops (Pittman *et al.*, 1996). A live-attenuated vaccine, TC-83, was developed by passage of Trinidad donkey (TrD) virus in guinea pig heart cells (Berge *et al.*, 1961; Lord, 1974; McKinney, 1972; Sharman, 1972). The murine response to TC-83 vaccination develops rapidly. Protection against subcutaneous or airborne challenge from virulent TrD virus occurs at 4 days post-vaccination, and is associated with production of VEEV neutralizing antibodies (Burke *et al.*, 1977; Ferguson *et al.*, 1978; Fillis & Calisher, 1979; Johnson & Martin, 1974; Walton & Johnson, 1972). TC-83 vaccine is not available for general human use (Phillpotts *et al.*, 2002).

VEEV contains two major surface glycoproteins, E1 and E2. Similar to *Sindbis virus*, the VEEV virion likely contains protein spikes organized as trimers of E1–E2 heterodimers (Parades *et al.*, 2001; Phinney *et al.*, 2000, Zhang *et al.*, 2002). We have previously used murine monoclonal antibodies (mAbs) to analyse the antigenic structure of the VEEV E2 glycoprotein. These studies identified six epitopes ($E2^{c–h}$) that form a critical viral neutralization domain (E2 aa 182–207). Anti-$E2^{c}$ mAbs, such as 3B4C-4, exhibit high virus neutralizing and protective activity by blocking virus attachment to cells (Roehrig *et al.*, 1988; Roehrig & Mathews, 1985). More recently, anti-$E2^{c}$ and anti-$E2^{g}$ mAbs (1A4A-1 and 1A3A-9, respectively) were shown to protect mice prophylactically from an aerosol challenge with VEEV and also to cure mice from infection 24 h after virus inoculation (Phillpotts *et al.*, 2002).

Based on the foregoing evidence, murine mAbs specific for epitopes within the critical neutralization domain may be useful in the prevention and treatment of VEEV infection in humans. However, rodent antibodies are highly immunogenic in humans and therefore limited in their clinical application. Antibody humanization is a process used to decrease the immunogenicity of rodent mAbs by replacing much of the murine amino acid sequence with human amino acids, while maintaining the original antigenic specificity. Clinical studies have indicated that humanized antibodies are less immunogenic than murine or chimeric antibodies and thus have more therapeutic potential (Hwang & Foote, 2005; Tsurushita *et al.*, 2005). We have humanized mAb 3B4C-4 by using phage display technology and rational antibody design (Kang *et al.*, 1991; McCafferty *et al.*, 1990; Rader *et al.*, 1998; Scott & Smith, 1990; Smith, 1985). The resultant humanized mAb, designated Hy4-26C (Hy4 IgG), maintained the important biological activities of 3B4C-4 and was able to passively protect mice before both intraperitoneal (i.p.) and intranasal (i.n.) challenge with virulent VEEV and to cure mice after i.p. challenge.

METHODS

Vectors, viruses and cells. Phage display vector pRL5, also known as pComb3X (Barbas *et al.*, 2001), was used for library construction and panning in the bacterial strain ER2537 (New England BioLabs) that suppresses an amber stop between the end of the heavy (H) chain and the phage coat protein III to generate Fab-phage. This same vector was used with non-suppressor strain Top10F′ (Invitrogen) to produce soluble Fab containing both HA-epitope and His6-purification tags. Hybridoma cell line 3B4C-4 has been described previously (Roehrig *et al.*, 1982). Stable cell lines expressing Hy4 IgG were made in 293 EBNA cells using the Effectene reagent (Qiagen) and selected with puromycin concentrations of 5.0 or 20.0 μg ml^{-1}.

The VEE complex viruses used in this study were TC-83 (variety 1AB), TrD (1AB), P676 (1C), 3880 (1D), Mena II (1E) and Everglades (EVE, strain Fe3-7C, subtype 2) and were from the Division of Vector-Borne Infectious Diseases (DVBID) stocks. Viruses grown in Vero or BHK-21 cells were purified by equilibrium density-gradient centrifugation (Obijeski *et al.*, 1976). Purified TC-83 virus, used for panning phage display libraries, was inactivated by treatment with 0.3 % β-propiolactone in 0.1 M Trizma, pH 9, for 48 h at 4 °C. Virus inactivation was verified by inoculation of Vero cell culture or 4-day-old sucking ICR mice (intracerebrally) and monitored for cytopathic effect or signs of illness, respectively. Inactivated virus was evaluated for preservation of important epitopes (Roehrig & Mathews, 1985).

cDNA cloning of Fab genes from murine mAb 3B4C-4. Total RNA isolated from 3B4C-4 hybridomas was used with First Strand cDNA kit (Boehringer Mannheim Biochemical) for the generation of oligo(dT)-primed cDNA. Forward primers were pooled into three or four mixes and then used in combination with the single reverse primer for either κ- or H-chain genes (Barbas *et al.*, 2001). The light (L) chain products were digested by *Sac*I/*Xba*I and cloned into pRL5, followed by *Xho*I/*Spe*I digestion and insertion of the H chains. The murine L and H chains in pRL5 were subjected to several rounds of phage display selection on TC-83 antigen to ease the identification of the correct chains, essentially as described previously (Rader *et al.*, 1998).

Generating chimeric 3B4C-4 H chain. The murine variable region was amplified by PCR from a plasmid containing murine Fab 3B4C-4 cDNA using a murine forward primer (MHyVH1) (Barbas *et al.*, 2001) and a chimeric H chain reverse primer annealing to the murine framework (FR) 4 region and having a tail of human constant H (CH)-chain region 1 (ChimHy4-B; Supplementary Table S1 available in JGV Online). The human CH1 domain was derived from a human Fab clone using PCR. The murine variable H (VH)-chain region and human CH1 domains were fused by overlap PCR and then cloned into pRL5. During the humanization process mAb amino acid composition was modified to include as many amino acids of human origin as possible, but maintaining the original epitope-binding specificity. Supplementary Table S2 (available in JGV Online) delineates the humanization steps and amino acid source found in intermediate Fab clones as well as in the final Hy4 Fab.

Humanization of the L chain. The murine L chain complementary determining region (CDR) 3 was grafted into a library of rearranged human κ L chains. Human bone marrow mononuclear cells were obtained from Poietics/BioWhittaker. RNA was isolated and first strand cDNA was made as described above. The first PCR reactions were set up with human κ variable region forward primers (FR1 specific primers; Barbas *et al.*, 2001), and reverse primers annealing at the end of FR3 containing a tail of 3B4C-4's CDR3 sequence [Hy4LCDR3b1, Hy4LCDR3b2, Hy4LCDR3b3 and Hy4LCDR3b4 (Supplementary Table S1 available in JGV Online)]. The resulting product included the front half of the humanized L chain library containing a portion of the 3B4C-4-specific CDR3. The back half of the humanized L chain library was generated using an FR4 forward primer Hy4LCDR3-F (Supplementary Table S1) containing a tail of 3B4C-4's CDR3 in combination with human κ constant region reverse primer CK1dX (Supplementary Table S1). A fusion PCR in which the CDR3 region provided the overlap was set up using these two halves of the L chain library, using the same protocol described above, with primers RSC-F (Supplementary Table S1) and CK1dX. The fusion PCR product was digested by *Sac*I/*Xba*I and ligated into vector pRL5, which already contained the 3B4C-4 chimeric H chain. A phage display library was created and panned on TC-83 antigen.

Humanization of the H chain. The murine H chain gene was compared to human germline sequences using the VBase database (http://vbase.mrc-cpe.cam.ac.uk/) to identify the nearest match, which were the VH1 gene at locus 1-f and the J-gene JH3a. The human germline amino acids were used at all positions except the following: (i) CDR3 was entirely murine amino acid sequence, (ii) where choice of murine or human CDR1 and CDR2 was given, or (iii) where choice of amino acid was given due to divergence of human and murine amino acid at selected FR positions [Vernier zone (Foote & Winter, 1992) or VH/variable L (VL)-chain interface (Santos & Padlan, 1998) at amino acid positions 37, 48, 67, 69, 71 and 91] (Kabat *et al.*, 1991).

The humanized H chain library was constructed using oligonucleotide-ligation assembly to create separate front- and back-halves of the VH-gene (Chalmers & Curnow, 2001; Sutton *et al.*, 1995). Each half of the gene contained a portion of the CDR2 that was used for full VH-gene assembly by overlap PCR. For cloning purposes, the assembled VH-genes contained an *Xho*I restriction site just prior to the +1 amino acid, as well as a small portion of the CH1 region containing the native *Apa*I restriction site. Oligonucleotides were synthesized and then combined so that six complementary sets could be assembled (Table 1). Each oligonucleotide set was separately added to reaction mixtures containing 1× ligation buffer and Ampligase Thermostable DNA Ligase (Epicentre Technologies). Reactions were thermocycled for 30 cycles of 95 °C for 30 s, 60 °C for 30 s, 65 °C for 15 min (decreasing 1.0 min per cycle to a minimum of 1 min); followed by 65 °C for 15 min.

The resulting six sets of ligated DNA were then combined in PCR assembly reactions to create four sets of humanized 3B4C-4 H chain inserts (combined sets 1+5, 2+6, 3+6, 4+5). The H chain PCR products were gel purified and then cloned by using *Xho*I/*Apa*I into three pRL5 vectors, each containing one of the humanized L chains (Hy4-11, Hy4-14 or Hy4-43). A resulting phage display library was panned on TC-83 antigen.

Modification of Hy4-26 L chain. DNA cassettes from FR1 to the beginning of FR3, created by Aptagen, incorporated human germline DPK9 sequence with bacterial codon preference, murine CDR1 and CDR2, as well as two positions of degeneracy where human germline and murine amino acid sequences differed in a Vernier zone and a VH/VL interface (Kabat #4 and #43). The two positions of choice resulted in a total of four cassettes. The cassettes were cloned into the L chain of Hy4-26 by using *Sac*I/*Ppu*MI. The four Fabs, and their corresponding amino acid at positions 4 and 43, were Hy4-26A (M4, P43), Hy4-26B (M4, A43), Hy4-26C (L4, A43) and Hy4-26D (L4, P43).

Conversion of Fab to IgG. The humanized Hy4-26C Fab was cloned into a single vector expression system that allowed mammalian expression of the respective whole IgG1/κ. The first step involved placement of mammalian control elements between the Fab L and H chains. The Fab sequences were then moved into the final IgG expression vector that contained all remaining elements necessary for expression in mammalian cells. DNA was sequenced to confirm construction of the desired Hy4 IgG plasmid.

Antibody purification. Fabs with HA-epitope and His6-purification tags were expressed in Top10F′ bacteria. Small-scale purification was done using supernatants from overnight bacterial cultures with the Ni-NTA Spin kit (Qiagen) under native conditions according to kit instructions. Large-scale Fab purifications were performed on a nickel charged HiTrap 5 ml chelating HP column (Amersham Biosciences) using the periplasmic fraction of 2 l overnight bacterial cultures. Complete Hy4 IgG antibody was expressed and purified from a stable 293 cell line. This cell line was adapted to serum-free medium and then grown in a hollow fibre system. IgG expressed in the culture supernatant was purified by fast protein liquid chromatography over a protein A column.

Mice. Swiss Webster mice were used for all passive antibody protection studies. The use of animals for research purposes complied with all relevant federal guidelines and specific protocols were approved by the DVBID Institutional Animal Care and Use Committee.

Enzyme immunoassays. Indirect ELISAs for assaying antiviral murine sera or humanized antibody were performed essentially as described previously (Roehrig *et al.*, 1980). Antibody binding to purified virus was detected by goat anti-species IgG-alkaline phosphatase (AP) conjugates (Jackson ImmunoResearch Laboratories). An absorbance ratio (A_{405} test sample/A_{405} negative control) of greater than two was considered to be positive. For the Fab ELISA, 200 ng inactivated TC-83 virus in 25 µl 0.1 M $NaHCO_3$ coating buffer, pH 8.6, was applied to one-half of the area of microtitre wells (CoStar High Bind; Corning). After overnight incubation at 4 °C the wells were blocked with 1 % BSA and bacterial supernatants were then added for 1 h at 37 °C. Subsequent 1 h incubations of anti-HA tag antibody 12CA5 (Roche Diagnostics) and anti-mouse IgG-AP conjugate (Sigma) were performed followed by addition of Sigma 104 substrate.

For the competition ELISA, purified Fabs containing an HA-epitope tag were used at a constant concentration corresponding to 80 % of maximum TC-83-binding activity. HA-tagged Fabs were mixed with increasing amounts of unlabelled competitor 3B4C-4 Fab. Binding of the HA-tagged Fabs to TC-83 virus was detected as described for the Fab ELISA.

Neutralization assays. The plaque-reduction neutralization test (PRNT) for complete IgG antibodies was done according to established protocols in Vero cells (Monath, 1976), using approximately 60–100 p.f.u. of TC-83 virus; end-point titres corresponded to a 70 % reduction in the number of plaques. The PRNT assay for Fab antibody fragments included an incubation with 0.025 ml of a secondary antibody, anti-mouse or anti-human $F(ab')_2$, for 30 min at 37 °C after incubation of the purified Fab (0.05 ml) virus (0.025 ml) mixture for 30 min at 37 °C (Mathews *et al.*, 1985). The secondary-antibody incubation was done to promote cross-linking of Fab fragments to enhance neutralization.

Passive antibody transfer and virus challenge in mice. For studies using prophylactic antibody treatment, 6–8-week-old Swiss Webster mice were inoculated i.p. with 100 µl of specified amounts of purified murine mAb 3B4C-4 or its humanized equivalent Hy4 IgG approximately 24 h prior to virus challenge. For therapeutic treatment, mice were inoculated i.p. with 10 µg mAb within 1 h of virus inoculation, or 24 or 48 h after virus infection. Mice were challenged i.p. with 100 mean morbidity doses (100 i.p. MD_{50}) of VEEV (TrD) (126 p.f.u.) or VEEV IC (P676) (126 p.f.u.). Hy4 IgG-treated mice were also challenged i.p. with either 10^3, 10^5 or 10^7 i.p. MD_{50} or intranasally (i.n.) with 100 i.n. MD_{50} (1350 p.f.u. in 5 µl BA-1) of VEEV (TrD) to determine antibody protective capacity. Mice were monitored for signs of illness for 2 weeks; survivors were bled 14 days post-challenge. Although some animals died as a result of virus challenge, death was not a required end point. Animals were euthanized when they became severely ill or paralysed.

To determine the amount of passive antibody remaining in the peritoneal cavity at the time of virus inoculation, groups of five mice inoculated i.p. with either 100, 10, 1 or 0.1 µg Hy4 IgG in 100 µl vols were subjected to peritoneal lavage with 7 ml PBS 24 h following antibody transfer. The recovered lavage fluid was clarified and an aliquot was concentrated 10 times by ultrafiltration (Millipore), and was monitored for Hy4 IgG binding to TC-83 virus by ELISA. Absorbance values (A_{405}) were compared to a standard curve of purified Hy4 IgG to estimate the amount of antibody in both lavage fluid and in total blood volume of animals given the same dose of mAb. The mean blood volume was estimated at 7.17 ml per 100 g body weight (Sluiter *et al.*, 1984). The mean body weight for 8-week-old female mice was 32.72 g; therefore, the mean blood volume was 2.35 ml.

Nucleotide sequence accession numbers. The nucleotide sequence data for murine mAb 3B4C-4 and humanized Hy4 IgG were assigned the following GenBank accession numbers: (i) 3B4C-4 murine L chain (DQ487205), (ii) 3B4C-4 murine V-gene and CH1 of H chain (DQ487206), (iii) Hy4-26C L chain (DQ487207) and (iv) Hy4-26C H chain (DQ487208).

RESULTS

Cloning and humanization of 3B4C-4

Cloning of murine hybridoma 3B4C-4 as a Fab was performed by RT-PCR recovery using a collection of different L and H chain family primers. The consensus sequence was determined and used as the murine 3B4C-4 hybridoma sequence. The L chain humanization was done so that only the murine CDR3 amino acid was retained (Rader *et al.*, 1998). This resulted in identification of three humanized L chain clones, Hy4-11, Hy4-14 and Hy4. For the H chain humanization, Fabs Hy4-26, -53 and -63 with three H chain murine CDRs were selected from a small rationally designed library.

A competition ELISA showed that the HA-tagged humanized Fabs Hy4-26, -53 and -63 were competed by unlabelled 3B4C-4 Fab for binding to TC-83 virus, indicating that they bound at or near the original 3B4C-4 epitope on the virus (data not shown). Purified Fabs Hy4-26, -53 and -63 were also analysed by Western blot on reduced and non-reduced TC-83 virus and, like the murine mAb, specifically recognized the viral E2 glycoprotein (data not shown).

Modification of Hy4-26 L chain and ELISA activity of the modified Hy4-26 Fabs

To determine if a humanized Fab retaining all six murine CDRs would have improved activity, modifications were made to the Hy4-26 L chain. In addition to grafting in murine CDR1 and CDR2, two positions of degeneracy were allowed on the L chain where human germline and murine amino acid sequences differed. This generated four versions of the modified L chain: Fabs Hy4-26A, Hy4-26B, Hy4-26C and Hy4-26D.

The modified Fabs were purified and tested for binding to TC-83 virus by ELISA (data not shown). Fab Hy4-26A, as a representative of the panel, was then tested in a competition ELISA against murine 3B4C-4 Fab for binding to TC-83 virus. The results showed that Fabs Hy4-26A and 3B4C-4 competed for the same (or similar) TC-83 virus epitope (Fig. 1).

Neutralization analysis of humanized Fabs

The virus-neutralizing activity of three humanized Fab clones (Hy4-26, -53 and -63) and four modified clones of Fab Hy4-26 (Hy4-26A, -26B, -26C and -26D) was tested by the secondary antibody-enhanced PRNT assay using TC-83 virus (Table 2). The Hy4-26 Fab and its four variants were able to neutralize virus efficiently compared with the unaltered, bacterially expressed murine 3B4C-4 Fab, indicating that for these Fabs the humanization process had not adversely affected this important biological function. Based on both the antigen-binding and neutralization results, Fab Hy4-26C was converted to whole IgG antibody for further study.

Antigenic specificity and neutralization activity of Hy4 IgG

The humanized Hy4 IgG produced by large-scale expression was evaluated serologically to determine its binding and neutralization activity on the VEEV subtypes and varieties known to react with murine mAb 3B4C-4 (Table 3). The Hy4 IgG ELISA and PRNT end-point titres on epizootic VEEV varieties 1AB and 1C demonstrated that the cross-reactivity pattern of 3B4C-4 had been generally maintained (Table 3) (Mathews & Roehrig, 1982; Roehrig & Mathews, 1985). Moreover, the

neutralization titres of the humanized IgG antibody were as high as those of its murine counterpart on these VEEV varieties. However, the reactivity of Hy4 IgG was much reduced for the enzootic VEEV variety 1D and subtype 2, both by ELISA and PRNT, compared with 3B4C-4, suggesting that some alteration in antibody reactivity had occurred during the humanization process.

Hy4 IgG clearance in mice

Clearance rates of 100 and 10 μg doses of Hy4 IgG administered i.p. to groups of 10 6–8-week-old mice were evaluated over a 2 week period by ELISA (Fig. 2). The 5 day clearance pattern was similar to that found for mAb 3B4C-4 given by the intravenous route (Mathews & Roehrig, 1982). Hy4 IgG was still detectable 2 weeks after transfer and the titre had decreased by a maximum of approximately threefold.

Protection of Swiss Webster mice from TrD virus challenge by prophylactic passive antibody transfer

Groups of 10 mice were inoculated i.p. with four different amounts of purified Hy4 IgG and challenged i.p. 24 h later with 100 i.p. MD_{50} of virulent TrD virus (Table 4). The survival rate was 70–100 % for the antibody-treated groups, including mice given as little as 100 ng Hy4 IgG ($P<0.05$, two-tailed Fisher's exact test). None of the control mice survived. The mean survival time (m.s.t.) for mice in the PBS control group was 6.0 days. Overall, just four mice died in two different antibody treatment groups, and these deaths occurred 6–10 days post-challenge. None of the surviving mice in any group, including those mice that mounted an anti-VEEV antibody response, showed signs of virus-induced morbidity (hair ruffling, ataxia). For comparison purposes, one group of mice was given 10 μg murine mAb 3B4C-4 and 90 % of these mice survived TrD virus challenge.

Passively protected mice were also challenged by the i.n. route 24 h after Hy4 IgG transfer with 100 i.n. MD_{50} of TrD virus (Table 4). Of mice inoculated with 500 μg of mAb 80 % survived the challenge. The dose of TrD virus, 1350 p.f.u., required for the i.n. challenge was nearly 11-fold greater than that used for i.p. challenge to produce similar morbidity in naïve mice. The m.s.t. for unprotected control mice that received an i.n. virus challenge was 4.9 days. Non-survivors that had an extended m.s.t. (6 days) were from the group that received 500 μg Hy4 IgG.

The survivor sera were tested by ELISA on TC-83 virus to determine the residual human antiviral IgG titre as well as to detect any *de novo* murine antibody response to the challenge virus (Table 4). The level of humanized IgG antibody remaining 2 weeks after challenge appeared to be generally related to the amount of antibody that was transferred, with $\log_{10}$ geometric mean titres (g.m.t.) ranging from a high of 3.83 to a low of 0.36 (corresponding to doses of 100 and 0.1 μg passive antibody, respectively). The titre of murine antiviral antibody was inversely related to the amount of the passive antibody dose; only those mice given the smallest dose of passive antibody (0.1 μg) had significant immune responses to the challenge virus (murine antiviral titres of ≥1:12800 for seven mice). For two mice inoculated with 100 μg Hy4 IgG, there was an apparent problem with the antibody transfer, since very little humanized IgG could be detected 14 days post-challenge (Table 4, row 2). These two mice evidently received enough passive antibody to allow them time to mount a significant protective antibody response ($\log_{10}$ g.m.t. of 5.05) and they showed no signs of illness. Based on the results of the titration of passive antibody (Table 4), a

standard dose of 10 μg Hy4 IgG was used for all subsequent passive antibody–virus challenge experiments. The capacity of a 10 μg dose of Hy4 to protect mice from a range of TrD virus challenge doses (10^2–10^7 i.p. MD_{50}) was examined (Table 5). Significant numbers of mice were protected at each virus challenge dose, up to the maximum possible challenge of 10^7 i.p. MD_{50} (2.26×10^7 p.f.u.) ($P<0.001$ for mAb-treated groups challenged with 10^2, 10^3 or 10^7 i.p. MD_{50} of TrD virus, and $P<0.005$ for the group challenged with 10^5 i.p. MD_{50} TrD virus). None of the surviving mice, even those that mounted an anti-VEEV response, showed any overt signs of illness. Survivor sera were evaluated by ELISA on TC-83 virus for both humanized and murine antiviral antibodies (Table 5). Residual Hy4 IgG was detected only in survivors given the two lowest virus challenge doses (10^2 and 10^3 i.p. MD_{50}); conversely, higher levels of induced murine antibodies were found only in survivors of the two highest virus challenge doses (10^5 and 10^7 i.p. MD_{50}). A 10 μg dose of Hy4 IgG, administered 24 h prior to virus challenge, was also able to protect significant numbers of mice from lethal infection with 100 i.p. MD_{50} of epizootic VEEV variety 1C (P676) (10 of 10 mice survived; $P<0.05$).

Peritoneal lavage performed on mice 24 h after i.p. transfer of three different amounts of Hy4 IgG revealed that the level of antibody detected in the peritoneal cavity was directly related to the dose of antibody inoculated. However, the amount of Hy4 IgG calculated for the total volume of lavage fluid was much less than that determined for the total blood volume. For the 100 μg mAb dose, 24 h after transfer, the titre in the lavage fluid was 1:212 compared with a blood titre of 1:11571; for the 10 μg mAb dose, lavage fluid titre was 1:28 and blood titre was 1:1264. No Hy4 IgG could be detected in the lavage fluid for the 0.1 μg dose. Based on the estimates of the total amount of antibody present, the antibody detected in the lavage fluid was only 1 % (about 1 μg) or 2–4 % (<1 μg), of that found in the total blood volume for the 100 μg mAb dose or the 10 μg dose, respectively.

Therapeutic administration of Hy4 IgG protected mice up to 24 h after TrD virus infection

Ten micrograms of humanized Hy4 IgG or murine mAb 3B4C-4 were inoculated i.p. at different times following TrD virus infection (100 i.p. MD_{50}) in Swiss Webster mice to determine the curative capacity of these antibodies (Table 6). mAb 3B4C-4 was able to protect mice if given within 1 h of virus challenge ($P<0.05$); however, Hy4 IgG was able to cure mice up to 24 h following TrD virus infection ($P<0.05$). Although some mice did survive virus infection when treated with 3B4C-4 24 h later, or with Hy4 IgG 48 h later, the numbers were not statistically significant. None of the surviving mice showed clinical signs of infection at any time during the 14 day observation period.

DISCUSSION

The current lack of a licensed human vaccine for VEEV advocates for evaluation of passive antibody immunization for prophylaxis or treatment of a VEEV infection. This strategy would be useful during a natural or intentional human outbreak, or following an occupational exposure to VEEV. The successful clinical use of well-characterized, neutralizing anti-VEEV murine mAbs requires circumventing a human anti-mouse antibody response. Therefore, we used antibody humanization as a strategy to generate protective, human compatible Fabs (Johnson *et al.*, 1997; Men *et al.*, 2004; Rader *et al.*, 1998, 2000; Santos & Padlan, 1998; Steinberger *et al.*, 2000).

The humanized Fab Hy4-26 had the best PRNT titres of the three clones tested. Modification of the L chain of Fab Hy4-26 was performed to further improve its activity, and these changes increased PRNT titres to within fourfold of 3B4C-4 Fab. Competition ELISA indicated that the humanized Fabs had retained the murine mAb's epitope specificity. After converting the modified Fab Hy4-26C to whole IgG, the resulting complete, humanized antibody contained about 9.7 % murine residues.

The association of *in vitro* neutralizing activity with *in vivo* protection for immunoglobulins used in passive immunization is well known (Parren & Burton, 2001). Hy4 IgG and murine mAb 3B4C-4 were nearly equal in concentration of antibody required to achieve a 70 % PRNT end point (Table 3). Although variation in testing protocols used by other investigators makes it difficult to directly compare antibody concentrations required to achieve *in vitro* neutralization end points, the 70 % PRNT end-point concentration of 39 ng ml^{-1} for Hy4 IgG makes this antibody one of the most effective compared with other antiviral recombinant humanized or human IgGs (Higo-Moriguchi *et al.*, 2004; Johnson *et al.*, 1997; Maruyama *et al.*, 1999; Men *et al.*, 2004; Tempest *et al.*, 1991; Tsui *et al.*, 1996). Hy4 IgG also demonstrated other desirable characteristics for a recombinant, humanized antibody: (i) retaining reactivity of parental mAb with epizootic VEEV strains, (ii) not enhancing virus replication nor interfering with the host immune response, and (iii) exhibiting protective capacity in an established animal model for VEEV infection.

The Hy4 IgG prophylactic and therapeutic capacities are the best indicator of its potential effectiveness for human use (Tables 4–6). This protective capacity was also shown to extend to the epizootic 1C VEEV variety. Hy4 IgG showed effective protection, even at a dose of 100 ng, versus a robust TrD virus challenge dose (Table 4). This result was not surprising since our previous studies with 3B4C-4 determined that no TrD virus was detected in most samples of spleen, brain or serum from passively protected mice on days 1 through 5 after virus challenge, nor was there any significant decrease in passive antibody levels in these tissues over the 5 day critical infectious period (Mathews & Roehrig, 1982).

Prophylactic Hy4 IgG also protected Swiss Webster mice from i.n. virus challenge, although a significantly higher antibody dose (500 μg) was required compared with that needed for protection from virus inoculated by the i.p. route (Table 4). This was likely due to the 10-fold higher dose of TrD virus needed for successful i.n. challenge versus i.p. challenge. The i.n. route also resulted in more rapid morbidity, with an m.s.t. of 4.9 days in untreated, control mice. Although Phillpotts *et al.* (2002) found that 100 μg of an anti-VEEV $E2^c$ mAb (1A4A-1) protected mice from aerosol challenge with TrD virus, the mice used were the inbred BALB/c strain, which may be more readily protected against an airborne

infection (Hart *et al.*, 1997). In Phillpotts study, 100 aerosol LD_{50} doses of TrD virus corresponded to approximately 89 p.f.u., a viral dose similar to that used in the current study (126 p.f.u.) for outbred mice, which were protected from i.p. VEEV challenge with a 100 μg dose of passive antibody.

Passive antibody persisted in the serum for at least 14 days, and the amount of Hy4 IgG remaining was generally related to the amount transferred and inversely related to the virus challenge dose (Tables 4 and 5). At the higher challenge doses (10^5 and 10^7 i.p. MD_{50}) no Hy4 IgG was detected after 14 days post-challenge, but significant numbers of mice were protected. This type of survival data versus increasing i.p. challenge doses were very similar to passive doses (5 and 20 μg) of murine 3B4C-4 mAb transferred by the intravenous route (Mathews & Roehrig, 1982). It is probable that the 10 μg dose of Hy4 IgG reduced the initial high viraemia and allowed mice to mount an effective response to the infection. This hypothesis is supported by the presence of high-titred murine antiviral antibody in day 14 sera from these survivors.

The failure to detect *de novo* murine IgG production in some protected animals suggests development of nearly complete sterilizing immunity following transfer of 1–100 μg Hy4 IgG with a challenge dose of 100 i.p. MD_{50} TrD virus, and 500 μg Hy4 IgG with a challenge of 100 i.n. MD_{50} TrD virus (Table 4). It is likely that there was substantial virus replication in mice immunized with 100 ng Hy4 IgG, as evidenced by the high-titred murine antiviral response. The amount of Hy4 IgG transferred ranged from a high of approximately 3 mg kg^{-1} body weight to a low of 3 μg kg^{-1}. Since significant protection from i.p. challenge was afforded at all antibody doses, Hy4 IgG compares favourably with anti-Ebola virus neutralizing recombinant mAb KZ52, which protected five of five guinea pigs from lethal Ebola Zaire virus challenge at 25 mg antibody kg^{-1} body weight, but protected only two of three animals at 5 mg kg^{-1} (Parren *et al.*, 2002). For mice challenged by the i.n. route, 15 mg antibody kg^{-1} body weight protected 80 % of the animals.

The persistence of Hy4 serum titre in mice for the 2 week observation period (Fig. 2) indicated that passive antibody could be an effective prophylactic for at least several weeks in mice. In human clinical trials, humanized antibodies have been shown to have reduced immunogenicity compared with murine mAbs and also to have prolonged serum half-lives (Nishibori *et al.*, 2006). Reported pharmacokinetics of a variety of humanized mAbs used in clinical trials reveal that serum half-life can vary from 6 to 7 days [HuOKT3 (Woodle *et al.*, 1999) and HuM195 (Caron *et al.*, 1998)] to longer than 20 days [rhuMAbVEGF (Gordon *et al.*, 2001) and epratuzumab (Leonard *et al.*, 2004)].

Only recently has it been reported that anti-VEEV mAbs can be used therapeutically to cure mice from an established VEEV infection (Phillpotts *et al.*, 2002). In our study, successful post-exposure antibody treatment with Hy4 IgG likely helped to control the initial course of infection, thus allowing the host to mount an effective immune response. It is probable that passive antibody could act at the level of the infected cell, as well as with free virus particles, to help control infection-related tissue damage (Parren *et al.*, 2002). The relatively short m.s.t. (5–6 days) of naïve mice infected with VEEV, as well as the development of peak virus titres in the brain within 3 days of peripheral infection, likely limits the window of opportunity for successful post-exposure treatment (Mathews & Roehrig, 1982). We were able to demonstrate, however, that passive antibody protected mice up to 24 h after infection (Table 6). Further evaluation of the protective power of this humanized antibody in non-human primates challenged with virulent VEEV will be necessary to establish clinical relevance of this approach.

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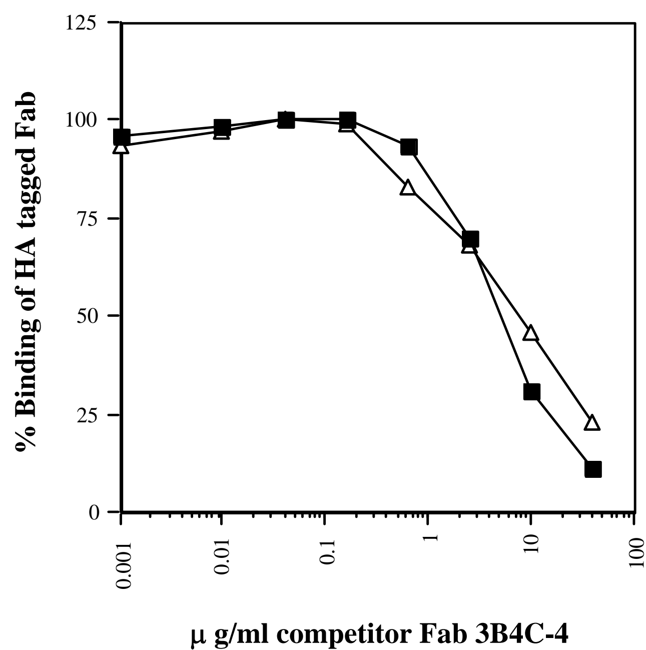

Fig. 1. Competition ELISA with humanized HA-tagged Fab Hy4-26A (△) or murine Fab 3B4C-4 (■) on TC-83 virus. HA-tagged Fab concentrations were held constant and were separately mixed with increasing concentrations of untagged competing 3B4C-4 Fab. HA-tagged Fab bound to virus was detected using an anti-HA secondary antibody.

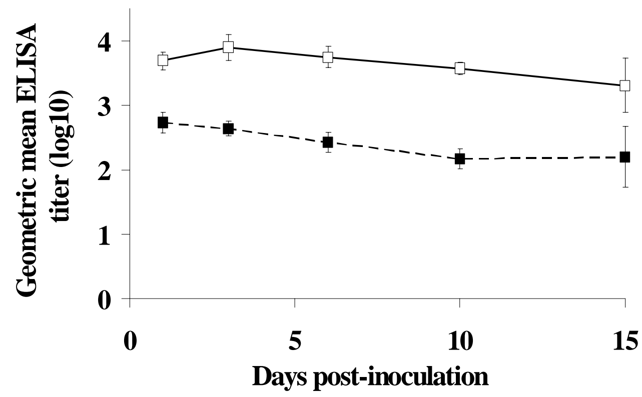

Fig. 2. Clearance of Hy4 IgG after intraperitoneal inoculation with 100 µg Hy4 IgG (□), (*n*=10); 10 µg Hy4 IgG (■), (*n*=7). Vertical bars represent standard deviations.

Table 1. Oligonucleotides for Hy4 humanized H chain library assembly and assembled oligonucleotide sets

Oligo	Oligonucleotide sequence (5′–3′)	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
1	GATCCGCTCGAGGTGCAGCTGGTGCAGTCCGGCGCTGAAGTGAAAAAACCGGGC	+*	+	+	+	−†	−
2	GCTACCGTGAAAATCTCCTGCAAAGTGTCCGGCTACACCTTCACCGACTAC	+	+	+	+	−	−
3H	TACATGCATTGGRTGCAGCAGGCTCCGGGCAAAGGCCTGGAATGGATSGGC	+	−	+	−	−	−
4H	CTGGTGGACCCGGAAGACGGCGAAACCATCTACGCTGAAAAATTCCAG	+	−	−	+	−	−
5	GGCCGCGYTACCMTCACCGMAGACACCTCCACCGACACCGCTTACATGGAA	−	−	−	−	+	+
6	CTGTCCTCCCTGCGCTCCGAAGACACCGCTGTGTACTWCTGCGCTCGCTCC	−	−	−	−	+	+
7	CTGACCTTCTTCGACGTGTGGGGCCAGGGCACCATGGTGACCGTGTCC	−	−	−	−	+	+
8	TCCGCCTCCACCAAGGGCCCATCGGTC	−	−	−	−	+	+
9	GACCGATGGGCCCTTGGTGGAGGCGGAGGACACGGTCACCATGGTGCCTG	−	−	−	−	+	+
10	GCCCCACACGTCGAAGAAGGTCAGGGAGCGAGCGCAGWAGTACACAGCGGT	−	−	−	−	+	+
11	GTCTTCGGAGCGCAGGGAGGACAGTTCCATGTAAGCGGTGTCGGTGGA	−	−	−	−	+	+
12H	GGTGTCTKCGGTGAKGGTARCGCGGCCCTGGAATTTTTCAGCGTAGATGGT	−	−	−	−	+	−
13H	TTCGCCGTCTTCCGGGTCCACCAGGCCSATCCATTCCAGGCCTTTGCC	+	−	−	+	−	−
14H	CGGAGCCTGCTGCAYCCAATGCATGTAGTAGTCGGTGAAGGTGTAGCCGGA	+	−	+	−	−	−
15	CACTTTGCAGGAGATTTTCACGGTAGCGCCCGGTTTTTTCACTTCAGCGCCGGA	+	+	+	+	−	−
16	CTGCACCAGCTGCACCTCGAGCGGATC	+	+	+	+	−	−
3M	TACATCAACTGGRTGCAGCAGGCTCCGGGCAAAGGCCTGGAATGGATSGGC	−	+	−	+	−	−
4M	CGCATCTACCCGGGCTACGGCAACACCAAATACAACGACAAATTCAAA	−	+	+	−	−	−
12M	GGTGTCTKCGGTGAKGGTARCGCGGCCTTTGAATTTGTCGTTGTATTTGGT	−	−	−	−	−	+
13M	GTTGCCGTAGCCCGGGTAGATGCGGCCSATCCATTCCAGGCCTTTGCC	−	+	+	−	−	−
14M	CGGAGCCTGCTGCAYCCAGTTGATGTAGTAGTCGGTGAAGGTGTAGCCGGA	−	+	−	+	−	−

*+, Oligonucleotide is included in the assembled set.

†−, Oligonucleotide is not included in the assembled set.

Table 2. *In vitro* neutralization end points for humanized 3B4C-4 Fab clones

Fab*	**PRNT end-point titre† (ng ml^{-1})**
Hy4-53	5000
Hy4-63	2500
Hy4-26	625
Hy4-26A	500
Hy4-26B	250
Hy4-26C	125
Hy4-26D	250
3B4C-4	125
Tetanus toxoid	>5000
3B4C-4 mAb	25

*Hy4-53, -63 and -26 are humanized Fab clones with different L chains; Hy4-26A–D are modified clones of Hy4-26. Tetanus toxoid Fab was used as a negative control; and 3B4C-4 mAb is a complete IgG molecule.
†PRNT, 70 % end point. This was a secondary-antibody-enhanced test that used anti-$F(ab')_2$ to cross-link Fabs.

Table 3. Serological reactivity of Hy4 IgG on VEEV varieties and subtypes

VEEV (variety or subtype)	**Hy4 IgG end-point titre**		**mAb 3B4C-4 end-point titre**
	ELISA (ng ml^{-1})	**PRNT* (ng ml^{-1})**	**PRNT (ng ml^{-1})**
Trinidad donkey (1AB)	1.95–3.1	39.4	70.8
TC-83 (1AB)	1.95–3.1	50	100
P676 (1C)	1.95	125	125–250
3880 (1D)	3910	>2 mg ml^{-1}	62500–125000
Mena II (1E)	125000	>1 mg ml^{-1}	>250000
Everglades (2)	250000	125000–250000	20000

*PRNT, 70 % end point.

Table 4. Protection of Swiss Webster mice from challenge with VEEV (TrD) following prophylactic administration of Hy4 IgG

Passive IgG*		VEEV (TrD) challenge†		Survivor sera ELISA g.m.t. versus TC-83 virus‡	
IgG	μg	Route	Survivors/total	Human IgG	Murine IgG
Hy4	100	i.p.	8/8	3.83 (0.14)	0.96 (0.34)
			2/2	0.15 (0.21)	5.05 (0.21)
Hy4	10	i.p.	10/10	2.13 (0.76)	1.09 (0.20)
Hy4	1	i.p.	7/10	2.12 (0.38)	1.26 (0.45)
Hy4	0.1	i.p.	9/10	0.36 (0.39)	4.42 (1.57)
PBS	–	i.p.	0/10	–	–
3B4C-4	10	i.p.	9/10	ND§	ND
Hy4	500	i.n.	8/10	3.53 (1.48)	0.94 (0.64)
Hy4	100	i.n.	0/10	–	–
Hy4	10	i.n.	0/10	–	–
PBS	–	i.n.	0/10	–	–
3B4C-4	10	i.n.	0/10	–	–

*Passive antibody was administered i.p. in 100 μl PBS to groups of 10 mice 24 h prior to virus challenge.
†Challenge dose of 100 i.p. MD_{50} in 100 μl BA-1 or 100 i.n. MD_{50} in 5 μl BA-1 was given 24 h after antibody transfer.
‡The survivors were bled 14 days post-challenge and the sera were tested against TC-83 virus by ELISA, detecting both humanized and murine antibody by using specific secondary antibody-enzyme conjugates. For the 100 μg i.p. IgG dose, data for two mice are reported separately since these mice had essentially no remaining humanized antibody but developed a significant murine antiviral response. Log_{10} geometric mean titres (g.m.t.) are shown with standard deviations in parentheses.
§ND, Not done.

Table 5. Titration of VEEV (TrD) challenge dose in Swiss Webster mice following prophylactic administration of Hy4 IgG

Hy4 IgG*	VEEV (TrD) challenge		Survivor sera ELISA g.m.t. versus TC-83†	
μg	Dose‡	Survivors/total	Human IgG	Murine IgG
10	10^2 i.p. MD_{50}	10/10	2.13 (0.76)	1.09 (0.20)
10	10^3 i.p. MD_{50}	18/20	1.61 (1.02)	2.13 (1.20)
10	10^5 i.p. MD_{50}	10/20	<1	≥4.13
10	10^7 i.p. MD_{50}	14/20	<1	≥4.64
PBS	10^2 i.p. MD_{50}	0/20	–	–

*Passive antibody was administered i.p. in 100 µl PBS to groups of 10 mice 24 h prior to virus challenge.
†The survivors were bled 14 days post-challenge and the serum was tested against TC-83 virus, detecting both humanized and murine IgG by using specific secondary antibody-enzyme conjugates. Log_{10} geometric mean titres (g.m.t.) are shown with standard deviations in parentheses.
‡Challenge dose was given i.p. in 100 µl BA-1 24 h after antibody transfer.

Table 6. Hy4 IgG administered therapeutically following VEEV (TrD) infection of Swiss Webster mice

A dose of 100 i.p. LD_{50} VEEV (TrD) was given i.p. in 100 µl BA-1.

Passive antibody* 10 µg		**Survivors/total (day 14)**
IgG	**Day**	
Hy4	0	9/10
Hy4	1	15/20
Hy4	2	3/10
3B4C-4	0	10/10
3B4C-4	1	7/20
PBS	0	1/10
PBS	1	0/10

*Passive antibody was administered i.p. in 100 µl PBS to groups of 10 mice within 1 h of virus infection (day 0), or 1 or 2 days later.