JOURNAL OF VIROLOGY, Dec. 2004, p. 12910–12918 Vol. 78, No. 23
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.23.12910–12918.2004

Chimpanzee Fab Fragments and a Derived Humanized Immunoglobulin G1 Antibody That Efficiently Cross-Neutralize Dengue Type 1 and Type 2 Viruses

Ana P. Goncalvez,[1] Ruhe Men,[1] Claire Wernly,[1] Robert H. Purcell,[2] and Ching-Juh Lai[1]*

Molecular Viral Biology Section[1] and Hepatitis Viruses Section,[2] Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Received 18 May 2004/Accepted 30 July 2004

Passive immunization with monoclonal antibodies from humans or nonhuman primates represents an attractive alternative to vaccines for prevention of illness caused by dengue viruses (DENV) and other flaviviruses, including the West Nile virus. In a previous study, repertoire cloning to recover Fab fragments from bone marrow mRNA of chimpanzees infected with all four DENV serotypes (dengue virus serotype 1 [DENV-1] to DENV-4) was described. In that study, a humanized immunoglobulin G1 (IgG1) antibody that efficiently neutralized DENV-4 was recovered and characterized. In this study, the phage library constructed from the chimpanzees was used to recover Fab antibodies against the other three DENV serotypes. Serotype-specific neutralizing Fabs were not identified. Instead, we recovered DENV-neutralizing Fabs that specifically precipitated the envelope protein and were cross-reactive with all four DENV serotypes. Three of the Fabs competed with each other for binding to DENV-1 and DENV-2, although each of these Fabs contained a distinct complementarity determining region 3 (CDR3)-H sequence. Fabs that shared an identical or nearly identical CDR3-H sequences cross-neutralized DENV-1 and DENV-2 at a similar high 50% plaque reduction neutralization test ($PRNT_{50}$) titer, ranging from 0.26 to 1.33 μg/ml, and neutralized DENV-3 and DENV-4 but at a titer 10- to 20-fold lower. One of these Fabs, 1A5, also neutralized the West Nile virus most efficiently among other flaviviruses tested. Fab 1A5 was converted to a full-length antibody in combination with human sequences for production in mammalian CHO cells. Humanized IgG1 1A5 proved to be as efficient as Fab 1A5 for cross-neutralization of DENV-1 and DENV-2 at a titer of 0.48 and 0.95 μg/ml, respectively. IgG1 1A5 also neutralized DENV-3, DENV-4, and the West Nile virus at a $PRNT_{50}$ titer of approximately 3.2 to 4.2 μg/ml. This humanized antibody represents an attractive candidate for further development of immunoprophylaxis against DENV and perhaps other flavivirus-associated diseases.

The four dengue virus (DENV) serotypes (DENV serotype 1 [DENV-1] to DENV-4) and several other arthropod-borne flaviviruses, including tick-borne encephalitis virus (TBEV), yellow fever virus, Japanese encephalitis virus (JEV), St. Louis encephalitis virus, and West Nile virus (WNV), are important human pathogens. Currently, DENVs are the most important in terms of morbidity and geographic distribution (16, 27). Patients with dengue usually develop fever, rash, and joint pain, and the disease is self limited. Occasionally, more severe forms of disease, known as dengue hemorrhagic fever and dengue hemorrhagic shock syndrome, also occur, especially in the regions of Southeast Asia where dengue is endemic and more recently in Central and South America. It is estimated that 50 to 100 million dengue infections and several hundred thousand cases of dengue hemorrhagic fever occur every year. *Aedes aegypti* and *Aedes albopictus* mosquitos are the principal vectors for human-to-human transmission of DENVs. Control of dengue epidemics by spraying of insecticides to reduce the vector mosquito population has proven to be rather ineffective. *Aedes* mosquito species are also responsible for transmission of WNV, which emerged for the first time in New York in 1999 (23). Since that time, the virus has spread widely to most of the continental United States. There were several thousand reported WNV infections in 2002, with a mortality of two hundred (32). Prevention of WNV infections has become an important public health issue in the United States and many other countries.

Dengue infection is thought to induce lifelong immunity against the same virus serotype. Cross-protection against other DENV serotypes (heterotypic immunity) in humans is brief, lasting only 2 to 9 months (36). Concurrent or sequential infections with different DENV serotypes are common (17, 22, 43). Epidemiological data suggest that a subsequent infection with a DENV serotype different from the serotype of the previous infection is more frequently associated with severe dengue illness than is the primary dengue infection. This observation has led to the hypothesis that immunopathological mechanisms involving the activities of DENV-specific antibodies or cytotoxic T cells contribute to dengue severity (19, 20, 21). However, evidence also indicates that dengue virulence could be in part due to a virus factor, such as replication

* Corresponding author. Mailing address: Molecular Viral Biology Section, Laboratory of Infectious Diseases, NIAID, NIH, Building 50, Room 6349, 50 South Dr., MSC 8009, Bethesda, MD 20892. Phone: (301) 594-2422. Fax: (301) 402-6413. E-mail: clai@niaid.nih.gov.

capacity (35, 42). To better protect against dengue infection and to minimize the risk of severe dengue, the current immunization strategy favors the use of a tetravalent vaccine against all four dengue serotypes. However, development of a safe and effective vaccine against dengue has been elusive.

In a previous study, an alternative strategy for the prevention of dengue fever by passive immunization with humanized antibodies was described (26). Repertoire cloning was employed to identify Fab antibody fragments from chimpanzees infected with all four DENV serotypes. One of these Fabs, 5H2, efficiently neutralized DENV-4 and was subsequently converted to a full-length immunoglobulin G (IgG) antibody containing human IgG sequences. Humanized antibody IgG 5H2 was produced in mammalian CHO cells and shown to neutralize DENV-4 at a 50% plaque reduction neutralization test ($PRNT_{50}$) titer of 0.03 to 0.05 μg/ml by a PRNT. With this success, the phage library constructed from the chimpanzee infected with multiple DENV serotypes was employed in an effort to recover Fab fragments against the other three DENV serotypes. In this study, we describe identification of Fab fragments that are broadly cross-reactive with all four DENVs as well as with other major insect-borne flaviviruses. Several of these Fabs were shown to cross-neutralize DENV-1 and DENV-2 at a similar high titer and DENV-3 or DENV-4 at a reduced titer. A full-length humanized IgG1 antibody, designated IgG1 1A5, was produced by combining Fab 1A5 with human IgG1 sequences. Humanized IgG1 1A5 antibody, like Fab 1A5, efficiently neutralized DENV-1 and DENV-2 but less efficiently neutralized DENV-3 and DENV-4, as well as other flaviviruses. Humanized antibody IgG1 1A5 represents an attractive candidate for further development of immunoprophylaxis against DENVs.

MATERIALS AND METHODS

Inoculation of chimpanzees with multiple DENV serotypes and preparation of lymphocytes from bone marrow. As described previously, two chimpanzees (numbers 1616 and 1618) that had been intrahepatically transfected with infectious RNA transcripts of a full-length DENV-4 cDNA clone (26) were infected subcutaneously 9.5 months later with a mixture of DENV-1 (Western Pacific strain), DENV-2 (New Guinea C strain; prototype) and DENV-3 (strain H87), each at 10^6 PFU, diluted in minimal essential medium (MEM) plus 0.25% human serum albumin. Twelve weeks after infection with the multiple DENV serotypes, bone marrow was aspirated from each chimpanzee, and the lymphocytes were prepared by centrifugation on a Ficoll-Paque gradient.

Construction of γ1/κ chimpanzee Fab antibody library. Repertoire cloning of chimpanzee Fab fragments was described earlier (26). Briefly, approximately 3×10^7 bone marrow lymphocytes from chimpanzee 1618, which developed higher neutralizing antibody titers against DENV-1, DENV-2, and DENV-3 than did chimpanzee 1616, were used for phage library construction. Total RNA from lymphocytes was extracted with the RNA Extraction kit (Stratagene, La Jolla, Calif.) and reverse transcribed with oligo(dT) as a primer with the ThermoScript RT-PCR system (Invitrogen). Chimpanzee V_L-C_L DNA sequences were amplified by PCR using seven pairs of human κ light-chain family-specific primers and a constant domain 3′ primer with AmpliTaq DNA polymerase (Perkin-Elmer) (2, 14, 34, 38). Chimpanzee V_H-C_{H1} DNA sequences were similarly amplified with nine human γ1 heavy-chain family-specific 5′ primers and a chimpanzee γ1-specific 3′ primer across the constant domain one-hinge junction (14, 38).

Pooled κ light-chain DNA fragments were digested with SacI and XbaI and then cloned into the pComb 3H vector by electroporation of eletrocompetent *Escherichia coli* XL-1 Blue (Stratagene). The recombinant plasmid was used for cloning of the pooled γ1 heavy-chain DNA fragments at the XhoI and SpeI sites. A library size of 2×10^8 to 4×10^8 colonies of transformed *E. coli* was obtained at each cloning.

Preparation of DENVs from infected mosquito C6/36 cells. Mosquito C6/36 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) plus gentamicin and amphotericin B (Fungizone). Confluent cells were infected with DENV-1, DENV-2, DENV-3, or DENV-4 of the strain indicated above, each at a multiple of infection (MOI) of 0.1 in MEM containing 2% FBS. DENV-1 (prototype Hawaii strain), kindly supplied by L. Rosen, and DENV-2 (New Guinea B strain), kindly supplied by W. Schlesinger, were also used. Infected cells were placed in serum-free medium (VP-SFM; Gibco Corp.) 1 day after infection and incubated at 28°C. The culture medium was harvested on days 6, 8, and 10 after infection, and fresh serum-free medium was added after each harvest. The virus titer in the medium was determined by a focus assay of Vero cells, and the medium was kept refrigerated for use as a panning antigen and for enzyme-linked immunosorbent assay (ELISA) and a neutralization assay.

Preparation of WNV/DENV-4 chimera, JEV, and LGTV. Vero cells were grown in MEM supplemented with 10% FBS plus gentamicin and Fungizone at 37°C. Confluent Vero cell monolayers were infected at an MOI of 1 with Langat virus strain TP 21 (LGTV), kindly supplied by R. Shope, or the WNV/DENV-4 chimera, kindly supplied by A. Pletnev. The infected cells were placed in MEM containing 2% fetal calf serum. JEV vaccine strain SA14-14-2, kindly supplied by K. Eckels and R. Putnak, was also propagated in Vero cells. The culture medium was harvested 7 days after infection, and titers were determined by a focus assay of Vero cells. For use as ELISA antigens, LGTV, JEV, and WNV/DENV-4 were grown in serum-free medium as described above. For neutralization assays, each of the above virus stocks was prepared in MEM containing 20% FBS and frozen until use.

Panning of phage library using DENV-1, DENV-2, or DENV-3 as an antigen. The pComb H DNA library that contained the V_L-C_L and V_H-C_{H1} inserts constructed earlier was again used for phage preparation. Identification of Fabs that were recovered from separate pannings against DENV-1, DENV-2, or DENV-3 was performed as described previously (26). Briefly, a bacterial culture with a diversity greater than 2×10^8 prepared by transformation with the plasmid DNA library was infected with VSC M13 helper phage (Stratagene) at an MOI of 50 to generate a phage display library. The phage library was panned by affinity binding on DENV-1, DENV-2, or DENV-3 virions captured by chimpanzee DENV-convalescent-phase sera coated on the wells of an ELISA plate. Following three cycles of panning, the selected phage was used for infection of *E. coli* XL-1 to produce phagemid DNA. Phagemid DNA was cleaved with SpeI and NheI to remove the phage gene III segment and circularized prior to use for transformation of *E. coli* XL-1. *E. coli* colonies were screened by ELISA to identify clones that yielded soluble Fab fragments reactive with DENV-1, DENV-2, or DENV-3.

DNA sequencing of DENV-specific Fab clones. Plasmids from selected *E. coli* clones producing soluble Fabs were first analyzed by digestion with BstN1 to identify clones with distinct cleavage patterns. Sequence analysis of the V_H and V_L DNA inserts was performed with an automated DNA sequencer using a *Taq* fluorescent dideoxynucleotide terminator cycle sequencing kit. The following primers were used: 5′ ACAGCTATCGCGATTGCAGTG and 5′ CACCTGAT CCTCAGATGGCGG for sequencing the V_L segments and 5′ ATTGCCTACG GCAGCCGCTGG and 5′ GGAAGTAGTCCTTGACCAGGC for sequencing the V_H segments. Software Vector NTi, suite 7.0 (InforMax), was used for analysis of the sequences. The DNAPLOT software program (MRC Center for Protein Engineering) was used for a homologous sequence search of the human IgG variable segments in the data bank.

Fab production and purification. Selected *E. coli* clones were grown in 2 liters of Luria broth containing 1% glucose, 100 μg of ampicillin/ml, and 10 μg of tetracycline/ml to an early log phase at 30 C. The bacteria were pelleted and resuspended in 2 liters of Luria broth containing ampicillin and tetracycline plus 0.1 mM inducer isopropyl-β-D-thiogalactopyranoside (IPTG) for growth at 30°C for 4 to 5 h (14, 38). After induction, the bacteria were collected and resuspended in 40 ml of buffer containing 50 mM phosphate buffer (pH 8.0), 10 mM Tris-HCl, 100 mM NaCl, and 0.1 mM protease inhibitor 4-(2-aminoethyl)-benzene sulfonyl fluoride. After three cycles of freezing and thawing to release the soluble Fab product from the bacterial periplasm, clear supernatant was prepared by centrifugation at 12,000 rpm in a Beckman JA-20 rotor for 60 min. The histidine-tagged Fab was affinity purified through a column of TALON metal affinity resin (Clontech). The purity of the Fab preparation was verified by polyacrylamide gel electrophoresis, and the Fab concentration was determined by ELISA with human IgG $F(ab')_2$ (Cappel) as a protein weight standard.

Biotinylation of purified Fab fragments and competition ELISA. Purified Fab was biotinylated with EZ-Link NHS-LC-Biotin (Pierce) as suggested by the supplier. After extensive dialysis against phosphate-buffered saline, the biotin-labeled Fab was analyzed for binding to DENV-1 or DENV-2 coated on wells of a microtiter plate. For competition ELISA, a fixed concentration of biotin-labeled Fab was mixed with the crude or purified preparation of competing Fabs in serial dilutions. The mixture was added to DENV-1- or DENV-2-coated wells

and incubated at 37°C. After washing was performed, streptavidin-alkaline phophatase (Pierce) was added for detection of biotinylated Fab reactive to DENV-1 or DENV-2 as previously described (26).

Western blot analysis. Virus samples were prepared by mixing approximately 10^5 PFU of each virus with an equal volume of 2× sample buffer containing 2% sodium dodecyl sulfate, 20% glycerol, 20 mM Tris-HCl (pH 8.0), and 0.03% bromophenol blue. The sample was loaded on a sodium dodecyl sulfate/-polyacrylamide gel and separated by electrophoresis. The gel was blotted on a nitrocellulose membrane, treated with 5% skim milk, and reacted first with Fab 1A5 and then with a 1/1,000 dilution of goat anti-human IgG-horseradish peroxides (Pierce). The blot was developed with Sigma Fast 3,3′-diaminobenzidine (Sigma-Aldrich).

Measurement of neutralizing activity of chimpanzee Fab fragments. Purified Fab antibodies were used in a PRNT to determine the neutralizing titer against each of the four DENV serotypes. Typically, approximately 50 PFU of the DENV in 100 μl of MEM was mixed with the same volume of the Fab in serial dilution. The DENV-Fab antibody mixture was incubated at 37°C for 1 h, and then 100 μl of the mixture was added to confluent Vero cells in a 24-well plate in duplicate. After an 1-h adsorption period at 37°C, an overlay of MEM containing 2% FCS and 1% tragacanth gum was added, and the plates were placed in a 5% CO_2 incubator at 37°C for 3 to 4 days. Virus foci that formed on the cell monolayer were immunostained (31). The $PRNT_{50}$ titer was calculated in micrograms per milliliter. Neutralization of the biosafety level 3 flaviviruses TBEV, JEV, and WNV was performed with attenuated biosafety level 2 variants LGTV, the JEV strain SA14-14-2, and the WNV/DENV-4 chimera, which contains the WNV preM-E structure protein genes on the DENV-4 backbone.

Construction of recombinant plasmid and expression of whole IgG1 molecules in CHO cells. The pFab cytomegalovirus (CMV)-*dhfr* vector for full-length IgG1 expression was constructed from plasmid pFab CMV originally obtained from P. Sanna (Scripps Research Institute) (37). A dihydrofolate reductase gene (*dhfr*) along with the transcription signals was inserted at the unique NotI site as a selecting marker and for gene copy amplification. In addition, an A-to-G substitution at the last nucleotide position of the intron that preceded the C_H3 exon present in the original vector was made to enable full-length IgG1 expression (26). Fabs 2H7, 1A5, 3A2, and 1B2 were selected for conversion to whole IgG1 antibodies for analysis of their neutralizing activity. The V_L DNA segment of each Fab was inserted into the expression vector at the SacI and XbaI sites. The V_H DNA segment of the Fab, regenerated by PCR, was then added at the XhoI and SpeI sites. The chimpanzee-specific sequences in the hinge region were converted to the human sequence as previously described (26).

Production of whole IgG molecules in CHO *dhfr* cells (ATCC) was carried out by transfection with RsrII-linearized plasmid in the presence of Lipofectamine (Gibco). Two days after transfection, cells in a T25 flask were replated in Iscove's modified Dulbecco medium supplemented with 10% FBS plus 10^{-7} M methotrexate in the absence of hypoxanthine-thymidine as the selecting medium (7, 45). Colonies of CHO cells resistant to 2×10^{-7} M methotrexate appeared approximately 2 weeks after transfection. The transformed CHO cells secreting IgG1 in the medium were identified after being cloned in a 96- or 24-well plate. To produce IgG1, the selected CHO cells were adapted to grow in CHO CD medium. The culture medium was concentrated, and the IgG1 product was purified through a protein A affinity column (Pierce). The apparent affinity constant (K_d) for the binding of the IgG to each of the four DENV serotypes was calculated as the antibody concentrations that gave 50% maximum binding by ELISA (24, 28).

RESULTS

Chimpanzee γ1/κ antibody library and identification of Fabs recovered by panning with DENV-1, DENV-2, or DENV-3. As described earlier, two chimpanzees (1616 and 1618) that had been intrahepatically transfected with infectious DENV-4 RNA were infected with a mixture of DENV-1, DENV-2, and DENV-3 9.5 months later. Each of the chimpanzees developed moderate to high $PRNT_{50}$ titers of antibodies against DENV-1, DENV-2, and DENV-3. The $PRNT_{50}$ titer against DENV-4 also increased appreciably after secondary dengue infection. Chimpanzee 1618 developed slightly higher neutralizing antibody titers against DENV-1, DENV-2, and DENV-3 than did chimpanzee 1616 (26). Previously, a phage library from bone marrow mRNA of chimpanzee 1618 was constructed and DENV-4 and dengue complex-specific Fabs were identified after panning of the library against DENV-4 (26). Based on these experiments, we reasoned initially that separate panning of the phage library using DENV-1, DENV-2, or DENV-3 would yield dengue-type, subcomplex, or complex-specific Fab clones that could be further analyzed for their capacity to neutralize DENV-1, DENV-2, or DENV-3 in vitro.

TABLE 1. DENV cross-neutralizing activities of Fabs identified by panning against DENV-1, DENV-2, or DENV-3

Fab[a]	Panning antigen	$PRNT_{50}$ titer (μg/ml) against:			
		DENV-1	DENV-2	DENV-3	DENV-4
2H7	DENV-1	0.26	0.33	5.92	7.26
2H5	DENV-1	0.47	0.53	20.8	9.26
1A5	DENV-2	0.49	0.77	3.49	4.23
1A10	DENV-2	0.94	5.26	26.3	12.6
1B2	DENV-2	0.50	3.13	>100	29.2
3A2	DENV-3	0.37	1.33	2.99	4.71
3E4	DENV-4	42.7	>100	>100	40.5

[a] Fabs which are underlined shared a similar V_H sequence or V_L sequence. Fab 3E4 was recovered from a chimpanzee after primary infection by panning against DENV-4, as described previously (26). Fab 3E4 was included for comparison with Fab 1A10.

Fab recovery. (i) Fabs recovered from panning against DENV-1. Several Fab clones with distinct BstNI digestion patterns were recovered following panning with DENV-1. A PRNT against DENV-1 was carried out to identify the most promising neutralizing Fab antibodies. Fab clones that did not neutralize DENV-1 or neutralized it only poorly were not studied further. Table 1 shows that Fab 2H7 and Fab 2H5 efficiently neutralized DENV-1 at a $PRNT_{50}$ titer of 0.26 and 0.47 μg/ml, respectively. Unexpectedly, each of these Fabs also neutralized DENV-2 at a titer similar to that detected for DENV-1. The $PRNT_{50}$ titer of these Fabs against DENV-3 or DENV-4 was reduced by 20 fold or more. Fab 2H5 and Fab 2H7 shared similar sequences (see below), but Fab 2H5 neutralized all four DENVs at lower titers than did Fab 2H7. Fab 2H5 was therefore not studied further.

(ii) Fabs recovered from panning against DENV-2. Three distinct neutralizing Fabs, i.e., 1A5, 1A10, and 1B2, were identified in this study (Table 1). Like Fab 2H7 and Fab 2H5 identified above, Fab 1A5 efficiently neutralized both DENV-1 and DENV-2 at a similar $PRNT_{50}$ of 0.49 and 0.77 μg/ml, respectively; it also neutralized DENV-3 and DENV-4, but at a lower titer. Fab 1B2 and Fab 1A10 neutralized DENV-1 more efficiently than DENV-2 and much more efficiently than DENV-3 or DENV-4.

(iii) Fabs recovered from panning against DENV-3. A large number of Fab clones showing a distinct BstN1 digestion pattern were recovered from the library by panning against DENV-3. Fab 3A2 neutralized DENV-1 and DENV-2 at a titer of 0.37 and 1.33 μg/ml, respectively, and also efficiently neutralized DENV-3 at a $PRNT_{50}$ titer of 3.0 μg/ml (Table 1). The ability of Fabs to cross-neutralize DENV-1 and DENV-2 at a similar high titer was a novel characteristic of several monoclonal antibodies, regardless of the DENV serotype used as the panning antigen.

Analysis of V_H and V_L sequences. The amino acid sequences in the V_L and V_H segment of six selected Fab antibodies are

FIG. 1. Amino acid sequences of Fabs. (A) sequences of the V_L κ light-chain segments; (B) sequences of the VH γ1 heavy-chain segments. FR, framework region. Dashes represent amino acid deletions; and identical amino acids are indicated by dots. The sequence of Fab 3E4 described previously (26) was included for comparison with that of Fab 1A10.

shown in Fig. 1. Fabs 2H7, 2H5, 1A5, and 3A2 were closely related, as an identical or nearly identical sequence was present in various framework segments or complementarity determining regions (CDRs) of the light chain or the heavy chain. Nevertheless, minor sequence variations (two or more amino acids) among them were present in other regions of the heavy chain as well as some regions of the light chain. These Fabs contained an identical or nearly identical 16-amino-acid sequence, which included two cysteines in the CDR3-H domain principally involved in antigen binding. The sequences of Fab 1B2 and Fab 1A10 were distinct and contained a CDR3-H sequence different from those of Fabs 2H7, 1A5, and 3A2. Table 2 shows the result of a homologous sequence search of human IgG germ line gene segments most related to the V_H or V_L segments of the selected six chimpanzee Fabs. The germ line origin was the same for Fab 2H7, 2H5, 1A5, or 3A2 and the homology with the most related human sequence was 82 to 94%, excluding the CDR3-H and CDR3-L regions.

TABLE 2. Sequence similarity between chimpanzee Fab antibodies and their most related human immunoglobulin homologs[a]

Chimpanzee Fab	V_H vs human homolog		Reference	V_L vs human homolog		Reference
	Family (gene)	% Identity		Family (gene)	% Identity	
2H7	VH3 (8-1B)	84	3	VKI (DPK9)	90	6
2H5	VH3 (8-1B)	85	3	VKI (DPK9)	86	6
3A2	VH3 (8-1B)	82	3	VKI (DPK9)	86	6
1A5	VH3 (8-1B)	85	3	VKI (DPK9)	86	6
1B2	VH4 (DP-78)	87	5	VKI (Va)	94	33
1A10	VH1 (DP-10)	86	41	VKII (A2b)	85	13

[a] The DNAPLOT program was used to search for the most homologous sequence of human germ line IgG genes in the database. The percent amino acid identity in the V_H or V_L segment, excluding the CDR-3 region, is indicated.

TABLE 3. Binding activities of Fab monoclonal antibodies to each of the four DENV serotypes as determined by ELISA[a]

Fab	ELISA titer of Fab binding to:			
	DENV-1	DENV-2	DENV-3	DENV-4
1A5	4.1	3.8	3.8	3.8
3A2	4.1	3.8	3.8	3.8
2H7	4.1	3.8	3.6	3.8
1B2	3.9	3.8	3.8	3.8
1A10	4.1	3.8	3.6	3.8
5H2	<1.0	<1.0	<1.0	3.8
3E4	4.0	3.8	3.6	3.8
1F2	<1.0	<1.0	<1.0	<1.0

[a] Microtiter plates were coated with DENV-1, DENV-2, DENV-3, or DENV-4 virions. Data are presented as $\log_{10}$ of the reciprocal dilution that gave an optical density reading twofold or higher than the background. DENV cross-reactive Fab 3E4- and DENV-4-specific Fab 5H2 were described previously (23). The starting concentration of each Fab was approximately 140 μg/ml. Chimpanzee Fab 1F2 was used as a negative control.

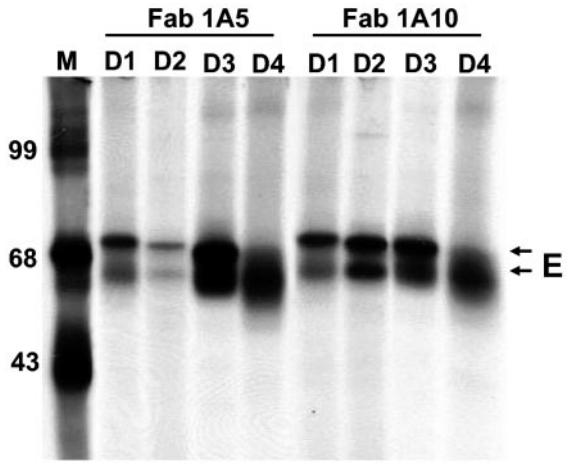

FIG. 2. Analysis of antigen specificity by radioimmunoprecipitation. Radioactive ^{35}S-methionine-labeled lysates separately prepared from Vero cells infected with each of the DENV serotypes (D1 to D4) were used for immune precipitation with Fab 1A5 or Fab 1A10. Lane M, molecular mass markers in kilodaltons. Each of the Fabs precipitated the E protein of each of four DENV serotypes. Note that the E protein often migrated as a doublet or a broad band, probably resulting from differences in glycosylation.

The V_H and V_L sequences of these Fab antibodies were also compared with the corresponding sequence of the Fab antibodies previously recovered by panning with DENV-4. Interestingly, Fab 1A10 and Fab 3E4 shared an identical V_H sequence with the exception of two amino acids: one in the FR1 region and the other in the CDR3 region (Fig. 1B). These two Fabs, however, differed appreciably in various regions of the V_L sequence (Fig. 1A). While the neutralizing activity of Fab 3E4 against DENV-1 and DENV-2 was low (titer greater than 42 μg/ml), Fab 1A10 neutralized DENV-1 and DENV-2 at a titer of 0.94 and 5.26 μg/ml, respectively.

Antigen specificity of chimpanzee Fabs. Soluble Fabs were analyzed for binding activity to each of the four DENV serotypes by ELISA. Table 3 shows that each of these Fabs was broadly cross-reactive for all four dengue serotypes and had a similar high binding titer. Surprisingly, none of the Fab antibodies recovered from panning with DENV-1, DENV-2, or DENV-3 reacted in a dengue type-specific manner.

Radioimmunoprecipitation was performed to determine the antigen binding specificity for each of the Fabs with a radiolabeled lysate of Vero cells infected with DENV-1, DENV-2, DENV-3, or DENV-4. Figure 2 shows a typical autoradiogram of the immune precipitate separated by polyacrylamide gel electrophoresis. Fabs 1A5 and 1A10 specifically precipitated the E protein, migrating as a doublet, of each of the four DENVs. Fabs 2H7, 3A2,and 1B2 also precipitated E from the lysate of each of the four DENV serotypes (data not shown).

Analysis of chimpanzee Fabs binding to DENV-1 or DENV-2 by competition ELISA. Fabs 1A5, 2H7, and 3A2 shared an identical or nearly identical CDR3-H sequence, whereas Fab 1A10 and Fab 1B2 each contained a distinct CDR3-H sequence. The relatedness of the binding sites for Fabs 1A5, 1A10, and 1B2 on DENV-1 or DENV-2 was analyzed by competition ELISA. Surprisingly, binding of affinity-purified, biotinylated Fab 1A10 to DENV-1 was competed by the unlabeled crude preparation of Fab 1B2 and Fab 1A5 (Fig. 3A). Similarly, binding of biotinylated Fab 1A5 to DENV-1 was competed by Fab 1B2 and Fab 1A10 and binding of Fab 1B2 in competition with Fab 1A5 and Fab 1A10 (Fig. 3B and C). When DENV-2 was tested, the binding competition patterns among these three Fabs were essentially identical to that seen with DENV-1 (Fig. 3D to F). As a control, chimpanzee Fab 1F2, which did not bind either DENV-1 or DENV-2, failed to compete with any of the labeled Fabs. Thus, the site that was occupied by Fab 1A5 overlapped with the site occupied by Fab 1B2 and Fab 1A10 on DENV-1, and on DENV-2 E. The Fab 1A5 binding site (epitope) on the DENV-2 E protein was mapped in a separate study (15).

Cross-reactivity of chimpanzee Fabs to WNV and other flaviviruses. In the course of this study, we found that the Fabs recovered in this study also reacted with the WNV/DENV-4 chimera at a high titer, as detected by ELISA (data not shown). Fab 1A5 was selected for analysis of binding to the four DENVs and other major flaviviruses. Western blot analysis (Fig. 4) showed that Fab 1A5 reacted relatively strongly with each of the four DENVs and WNV/DENV-4. By comparison, Fab 1A5 bound weakly to JEV SA14-14-2 and LGTV TP 21. The reduced binding activity of Fab1A5 to the JEV SA14-14-2 and LGTV TP 21 reflected the low $PRNT_{50}$ titer (>70 μg/ml) of Fab 1A5 against these two viruses. Interestingly, Fab 1A5 neutralized WNV/DENV-4 chimera at a $PRNT_{50}$ titer of 4.8 μg/ml, similar to that measured for DENV-3 and DENV-4.

Production and characterization of full-length humanized IgG1 antibodies. With the exception of Fab 1A10, the Fab fragments were each converted to the full-length IgG1 antibody in combination with the human IgG1 sequence with the expression vector pFab CMV-*dhfr* for transformation of CHO cells (26). Among these antibodies, IgG1 1A5 was produced in the highest yield, approximately 2 μg/10^6 cells per day in the medium of the transformed CHO cells. IgG1 1A5 was selected to determine the $PRNT_{50}$ against each of the four DENVs (Fig. 5). IgG1 1A5 neutralized DENV-1 and DENV-2 at a $PRNT_{50}$ titer of 0.48 and 0.95 μg/ml, respectively. IgG1 1A5 also neutralized DENV-3 and DENV-4 at a $PRNT_{50}$ titer of 3.2 and 4.3 μg/ml, respectively. The apparent affinity constants determined by ELISA, termed ELISA K_d, were calculated at

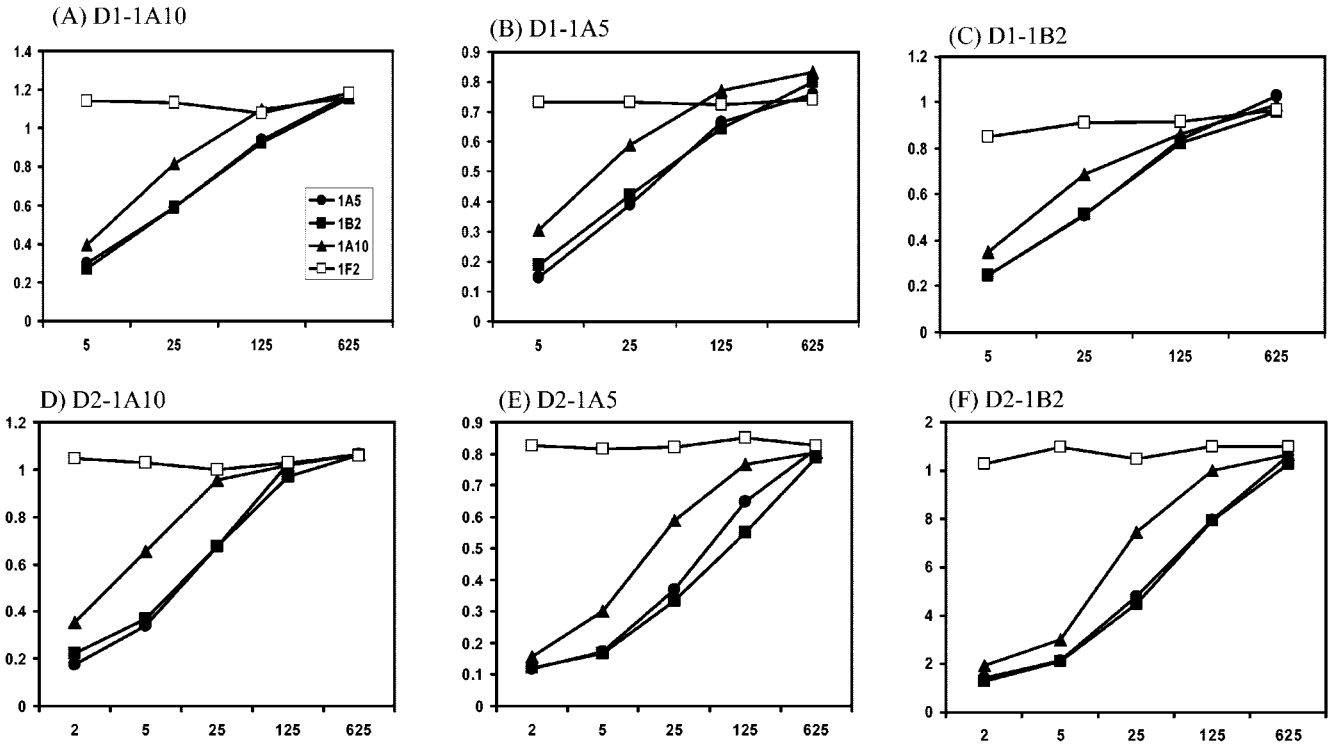

FIG. 3. Analysis of Fab binding to DENV-1 or DENV-2. Fabs 1A5, 1B2, and 1A10 were affinity purified, biotinylated, and used for analysis of binding activity to DENV-1 or DENV-2 by competition ELISA in the presence of competing, unlabeled Fabs. Chimpanzee Fab 1F2, which did not react with any of the DENVs, was used as a negative control. The numbers on the *y* axes are optical density readings, and the *x* coordinates represent reciprocal dilutions of the competing Fabs. At the top of each panel, D1 or D2 indicates whether DENV-1 or DENV-2 was used. The insert inside panel A shows the symbol for each Fab; the symbols are the same for all six panels.

0.50, 0.60, 0.67, and 0.82 nM for DENV-1, DENV-2, DENV-3, and DENV-4, respectively, in the same decreasing order as the $PRNT_{50}$ titers against these viruses. Humanized IgG1 1A5 was also tested for neutralization of WNV/DENV-4, JEV strain SA14-14-2 and LGT strain TP 21 by PRNT. The $PRNT_{50}$ titer against WNV/DENV-4 was 3.8 μg/ml, whereas the $PRNT_{50}$ titer against JEV strain SA14-14-2 and LGTV strain TP 21 was 21 and 28 μg/ml, respectively (Fig. 5).

DISCUSSION

A safe and effective vaccine for prevention of DENV infection is still not available. Several tetravalent live-attenuated DENV vaccine formulations are currently in the late stages of development (8, 18). However, the safety of such DENV vaccines remains a concern. Passive immunization with neutralizing antibodies represents an attractive alternative to a DENV vaccine. Toward this goal, chimpanzees that had been intrahepatically inoculated with DENV-4 RNA and seroconverted from an earlier experiment were subsequently infected with a mixture of DENV-1, DENV-2, and DENV-3 to increase the repertoire of DENV-specific antibodies. The interval of 9.5

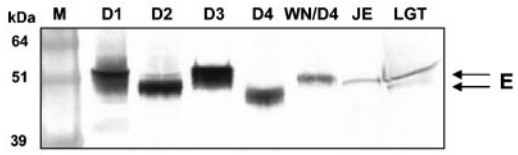

FIG. 4. Binding of Fab 1A5 to DENVs and other flaviviruses as measured by Western blotting. Approximately 10^5 PFU of each virus was applied and separated by polyacrylamide gel electrophoresis. Lanes: D1, DENV-1 strain Hawaii; D2, DENV-2 strain New Guinea B; D3, DENV-3 strain H87; D4, DENV-4 strain 814669; WN/D4, WNV/DENV-4 chimera; JE, JEV strain SA14-14-2; LGT, LGTV strain TP 21. The position of the E protein is indicated. Molecular mass markers are shown on the left, in kilodaltons.

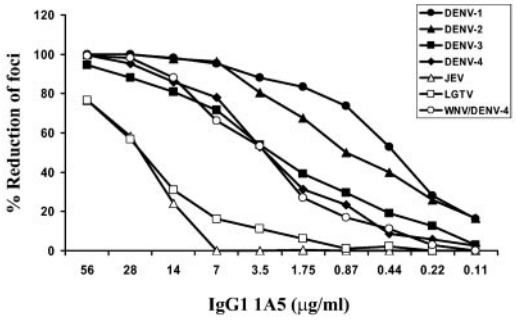

FIG. 5. In vitro neutralization of DENVs and other flaviviruses by humanized IgG1 1A5. The neutralizing activity of IgG1 1A5 against DENV-1 strain Hawaii, DENV-2 strain New Guinea B, DENV-3 strain H87, DENV-4 strain 814669, JEV vaccine strain SA14-14-2, LGTV strain TP 21, and the WNV/DENV-4 chimera was analyzed by a PRNT.

months between infections was apparently sufficient for heterotypic DENV immunity to wane, as both chimpanzees developed an antibody response to each of these viruses (26). Interestingly, earlier dengue research in humans also showed that heterotypic immunity against other DENV serotypes was brief, lasting 2 to 9 months (36).

Repertoire cloning of bone marrow mRNA from chimpanzees infected with all four DENV serotypes has been performed to identify Fab antibody fragments that could be used for development of clinically acceptable neutralizing antibodies. In an earlier study, serotype-specific Fab antibodies that efficiently neutralized DENV-4 were identified with DENV-4 as panning antigen. This success prompted us to use the same library for recovery and identification of neutralizing Fab antibodies against each of the other three DENV serotypes. A large panel of Fabs was recovered by panning of the phage library with DENV-1, DENV-2, or DENV-3. Regardless of the panning antigen used, recovered Fabs were shown to be cross-reactive to all four DENV serotypes, and serotype-specific neutralizing Fabs against DENV-1, DENV-2, or DENV-3 were not identified. Several Fab antibodies, as exemplified by Fabs 1A5, 2H7, and 3A2, were highly efficient for cross-neutralization against DENV-1 and DENV-2. Each of these Fab antibodies also neutralized DENV-3 and DENV-4, but at a reduced titer. These chimpanzee Fabs appear to represent a novel class of dengue complex cross-neutralizing monoclonal antibodies that have not been described before for mouse monoclonal antibodies.

It is reasonable to assume that the responses of the chimpanzees to primary infection with DENV-4 and to secondary dengue infection with a mixture of DENV-1, DENV-2, and DENV-3 are represented in the respective genetic repertoire of the phage libraries. A number of dengue cross-reactive Fab antibodies were identified from chimpanzee 1618 following primary infection, although their neutralizing activities were low or not detected (26). The secondary infection may have selected for the production of cross-reactive antibodies in the chimpanzee because of memory T cells and B cells. Evidence for such immune responses is provided by comparison of the sequences between Fab 3E4, recovered from primary infection with DENV-4, and Fab 1A10 recovered from the same chimpanzee after secondary infection with DENV-1, DENV-2, and DENV-3. Fab 3E4 and Fab 1A10 shared a nearly identical V_H sequence, although their V_L sequences varied. Fab 3E4 was cross-reactive to all four serotypes and was poorly neutralizing against each of these viruses (26). In contrast, Fab 1A10 neutralized DENV-1 and DENV-2 at a titer much higher than that measured for Fab 3E4 (Table 1). It appears that the B cell clone producing this antibody raised in the initial infection with DENV-4 was stimulated during the subsequent infection with a mixture of three other DENV serotypes. This situation is reminiscent of the antibody response termed original antigenic sin, first described for influenza infections (44).

The sequence similarity among Fabs 1A5, 2H7, 2H5, and 3A2 reflects their neutralizing activities against each of the four DENV serotypes. Characteristically, each of these Fabs contains a 16-amino-acid sequence in CDR3-H, which is longer than the sequence of 8 to 14 residues found in most human CDR3-H or the sequence of 8 to 12 residues in mouse CDR3-H sequences (46). Also, there are two Cys residues separated by four amino acids in the chimpanzee CDR3-H sequences. It is speculated that the two additional Cys could form a local disulfide bond, as all other Cys residues in Fab fragments or full-length antibodies participate in disulfide bond formation. Formation of this additional disulfide bridge could impose an additional constraint on the flexibility of the CDR3-H loop.

Despite their sequence differences, Fab 1A10, Fab 1B2, and Fab 1A5 competed with each other for binding to DENV-1 and DENV-2 in competition ELISA. This suggests that the binding sites of these apparently different Fabs on these viruses are spatially close or overlapping. It is also possible that the binding site on the surface of DENV-1 and DENV-2 E for each of these Fabs is unique, but binding of one Fab results in alteration of the binding sites for others through a steric hindrance. Interestingly, Fab 1A10 and Fab 1B2 neutralized DENV-1 more effectively than DENV-2 even though they were derived by panning against DENV-2.

Sequence analysis indicates that there is a high degree of similarity between the chimpanzee V_H and V_L sequences and their human germ line homologs (82% or greater). The sequence homology between humans and chimpanzees in the constant regions C_H1 and C_L is even greater, approaching 100% (9, 11). The high level of antibody sequence similarity suggests the possibility that the humanized chimpanzee antibodies may be administered directly to humans without further modifications. Experimental data available indicate that little immunogenicity is seen when components of human antibodies are introduced into rhesus monkeys or chimpanzees (10, 30).

It was reported in a previous study that the full-length humanized antibody IgG1 5H2 has a DENV-4-neutralizing $PRNT_{50}$ titer of 0.03 to 0.05 μg/ml, compared to the titer of 0.24 to 0.52 μg/ml measured for Fab 5H2 (26). On a molar basis, the neutralizing activity of IgG1 5H2 is approximately 30- to 40-fold higher than that of the Fab 5H2 fragment. A similar increase of neutralizing activity for mouse monoclonal antibody 4E11, compared to its derived Fab fragment against DENV-1, has been reported (40). A comparison of the neutralizing activities for several influenza virus HA monoclonal antibodies and their Fab fragments derived by papain cleavage has also been reported (39). This study has shown that most IgGs have a >90-fold increase of neutralizing activities compared to their Fabs. Based on these data, we had hoped that the full-length IgG1 1A5 antibody would have a neutralizing titer 30- to 40-fold higher than the Fab 1A5 fragment against DENV-1 or DENV-2. This turned out not to be the case, as there was only a threefold increase in neutralizing activity for IgG 1A5 over Fab 1A5 against each of the four DENV serotypes on a molar basis. A similar level of increase of activity has also been reported for the full-length IgG1 converted from a neutralizing Fab against the Ebola virus (25). The relative neutralizing activity between the monovalent Fab and bivalent IgG is probably dependent on accessibility to the epitope of the virus it recognizes.

The cause of severe dengue, which is sometimes associated with secondary infection and sometimes with primary infection, remains controversial. According to one hypothesis, the increased dengue severity is an immunopathological phenomenon caused by antibody-dependent enhancement of infection (19, 20). In a secondary infection, DENV could complex with

a subneutralizing concentration of cross-reactive antibodies produced during the primary infection, leading to an increased uptake and replication in susceptible mononuclear cells via their Fc receptors. This issue is particularly important for dengue prophylaxis with antibodies such as IgG1 1A5 in view of their cross-reactivity to all four DENV serotypes. Several FcRγ receptors have been identified on the cell surface, and their binding sites on the respective IgG have been identified (1, 4). It may be possible to diminish the binding affinity between the Fc portion of the antibody and cellular receptors by altering the FcRγ1 binding sequences in the IgG1 1A5. It remains to be determined if such mutations in the FcR binding sites would affect the DENV-neutralizing activity or the stability of the antibody product.

Antibody IgG1 1A5 could be a possible candidate for the development of a passive immunization strategy against dengue. One of the attractions is the use of IgG1 1A5 for cross-neutralization of DENV-1 and DENV-2. Administration of a dose of 2 mg of the antibody per kg of body weight would give a serum titer of approximately 40 and 20 $PRNT_{50}$ against DENV-1 and DENV-2, respectively. In addition, IgG1 1A5 also neutralized DENV-3, DENV-4, and WNV at a similar $PRNT_{50}$ titer, ranging from 3.2 to 4.2 μg/ml. WNV is becoming increasingly important for the public health of the United States and many other countries. It has been shown in a mouse model that passive administration of immune mouse sera or human immune gamma globulin protected against lethal WNV challenge (12). Administration of human gamma globulin also improved the clinical outcome after the virus had disseminated in the central nervous system in mice. Humanized IgG1 1A5 may therefore prove to be a valuable candidate not only for prophylactic but also for therapeutic application against this virus.

It should be pointed out that the neutralizing activity of Fab 1A5 and IgG1 1A5 against JEV strain SA14-14-2 and LGTV strain TP 21 was lower than that against WNV/DENV-4. The neutralizing titers of IgG 1A5 against wild-type JEV and other members of the TBEV remain to be determined. The amino acid sequence of the attenuated JEV strain SA14-14-2 differs from that of its parental virulent virus in a number of positions, including several in E (29). In another study (15), epitope mapping of Fab 1A5 by analysis of DENV-2 antigenic variants showed that a determinant responsible for antibody binding was localized to Gly_{106} within the flavivirus-conserved fusion loop in domain II of DENV-2 E. The results of that study provide evidence for an explanation that the reduced neutralizing activities of Fab 1A5 against these viruses stem from the sequence variations at or near the mapped determinant.

ACKNOWLEDGMENTS

We thank Max Shapiro and other members of Bioqual, Inc., for providing animal care, Kenneth Eckels and Robert Putnak for providing various dengue serotype viruses and the attenuated JEV SA14-14-2 strain, Robert Shope for providing Langat virus TP 21, Alexander Pletnev for providing the West Nile/dengue virus type 4 chimera, and Lynn Rasmussen for nucleotide sequencing.

REFERENCES

1. **Allen, J. M., and B. Seed.** 1989. Isolation and expression of functional high-affinity Fc receptor complementary DNAs. Science **243:**378–381.
2. **Barbas, C. F., III, A. S. Kang, R. A. Lerner, and S. J. Benkovic.** 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. USA **88:**7978–7982.
3. **Berman, J. E., S. J. Mellis, R. Pollock, C. L. Smith, H. Suh, B. Heinke, C. Kowal, U. Surti, L. Chess, C. R. Cantor, and F. W. Alt.** 1988. Content and organization of the human Ig VH locus: definition of three new VH families and linkage to the Ig CH locus. EMBO J. **7:**727–738.
4. **Chappel, M. S., D. E. Isenman, M. Everett, Y. Y. Xu, K. J. Dorrington, and M. H. Klein.** 1991. Identification of the Fc gamma receptor class I binding site in human IgG through the use of recombinant IgG1/IgG2 hybrid and point-mutated antibodies. Proc. Natl. Acad. Sci. USA **88:**9036–9040.
5. **Chothia, C., A. M. Lesk, E. Gherardi, I. M. Tomlinson, G. Walter, J. D. Marks, M. B. Llewelyn, and G. Winter.** 1992. Structural repertoire of the human VH segments. J. Mol. Biol. **227:**799–817.
6. **Cox, J. P., I. M. Tomlinson, and G. Winter.** 1994. A directory of human germ-line V kappa segments reveals a strong bias in their usage. Eur. J. Immunol. **24:**827–836.
7. **Dorai, H., and G. P. Moore.** 1987. The effect of dihydrofolate reductase-mediated gene amplification on the expression of transfected immunoglobulin genes. J. Immunol. **139:**4232–4241.
8. **Edelman, R., S. S. Wasserman, S. A. Bodison, R. J. Putnak, K. H. Eckels, D. Lang, N. Kanesa-Thasan, D. W. Vaughn, B. L. Innis, and W. Sun.** 2003. Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. Am. J. Trop. Med. Hyg. **69**(6 Suppl.)**:**48–60.
9. **Ehrlich, P. H., Z. A. Moustafa, K. E. Harfeldt, C. Isaacson, and L. Ostberg.** 1990. Potential of primate monoclonal antibodies to substitute for human antibodies: nucleotide sequence of chimpanzee Fab fragments. Hum. Antibodies Hybridomas **1:**23–26.
10. **Ehrlich, P. H., Z. A. Moustafa, J. C. Justice, K. E. Harfeldt, and L. Ostberg.** 1988. Further characterization of the fate of human monoclonal antibodies in rhesus monkeys. Hybridoma **7:**385–395.
11. **Ehrlich, P. H., Z. A. Moustafa, and L. Ostberg.** 1991. Nucleotide sequence of chimpanzee Fc and hinge regions. Mol. Immunol. **28:**319–322.
12. **Engle, M. J., and M. S. Diamond.** 2003. Antibody prophylaxis and therapy against West Nile virus infection in wild-type and immunodeficient mice. J. Virol. **77:**12941–12949.
13. **Feeney, A. J., M. J. Atkinson, M. J. Cowan, G. Escuro, and G. Lugo.** 1996. A defective V_κ A2 allele in Navajos which may play a role in decreased susceptibility to *Haemophilus influenzae* type b disease. J. Clin. Investig. **97:** 2277–2282.
14. **Glamann, J., D. R. Burton, P. W. Parren, H. J. Ditzel, K. A. Kent, C. Arnold, D. Montefiori, and V. M. Hirsch.** 1998. Simian immunodeficiency virus (SIV) envelope-specific Fabs with high-level homologous neutralizing activity: recovery from a long-term-nonprogressor SIV-infected macaque. J. Virol. **72:** 585–592.
15. **Goncalvez, A. P., R. H. Purcell, and C.-J. Lai.** 2004. Epitope determinants of a chimpanzee Fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to fusion loop of the dengue type 2 virus envelope glycoprotein. J. Virol. **78:**12919–12928.
16. **Gubler, D. J.** 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. **11:**480–496.
17. **Gubler, D. J., G. Kuno, G. E. Sather, and S. H. Waterman.** 1985. A case of natural concurrent human infection with two dengue viruses. Am. J. Trop. Med. Hyg. **34:**170–173.
18. **Guirakhoo, F., K. Pugachev, Z. Zhang, G. Myers, I. Levenbook, K. Draper, J. Lang, S. Ocran, F. Mitchell,. M. Parsons, N. Brown, S. Brandler, C. Fournier, B. Barrere, F. Rizvi, A. Travassos, R. Nichols, D. Trent, and T. P. Monath.** 2004. Safety and efficacy of chimeric yellow fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J. Virol. **78:**4761–4775.
19. **Halstead, S. B.** 1979. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J. Infect. Dis. **140:**527–533.
20. **Halstead, S. B.** 1988. Pathogenesis of dengue: challenges to molecular biology. Science **239:**476–481.
21. **Kurane, I., B. L. Innis, S. Nimmannitya, A. Nisalak, A. Meager, J. Janus, and F. A. Ennis.** 1991. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. J. Clin. Investig. **88:**1473–1480.
22. **Laille, M., V. Deubel, and F. F. Saite-Marie.** 1991. Demonstration of current dengue 1 and dengue 3 infection in six patients by the polymerase chain reaction. J. Med. Virol. **34:**51–54.
23. **Lanciotti, R. S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara, and D. J. Gubler.** 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science **286:**2333–2337.
24. **Lin, C.-W., and S.-C. Wu.** 2003. A functional epitope determinant on domain III of the Japanese encephalitis virus envelope protein interacted with neutralizing antibody combining sites. J. Virol. **77:**2600–2606.
25. **Maruyama, T., L. L. Rodriguez, P. B. Jahrling, A. Sanchez, A. S. Khan, S. T. Nichol, C. J. Peters, P. W. Parren, and D. R. Burton.** 1999. Ebola virus can

be effectively neutralized by antibody produced in natural human infection. J. Virol. **73:**6024–6030.
26. **Men, R., T. Yamashiro, A. P. Goncalvez, C. Wernly, D. J. Schofield, S. U. Emerson, R. H. Purcell, and C. J. Lai.** 2004. Identification of chimpanzee Fab fragments by repertoire cloning and production of a full-length humanized immunoglobulin G1 antibody that is highly efficient for neutralization of dengue type 4 virus. J. Virol. **78:**4665–4674.
27. **Monath, T. P.** 1994. Dengue: the risk to developed and developing countries. Proc. Natl. Acad. Sci. USA **91:**2395–2400.
28. **Moore, J. P., Y. Cao, L. Qing, Q. J. Sattentau, J. Pyati, R. Koduri, J. Robinson, C. F. Barbas III, D. R. Burton, and D. D. Ho.** 1995. Primary isolates of human immunodeficiency virus type I are relatively resistant to neutralization by monoclonal antibodies to gp120, and their neutralization is not predicted by studies with monomeric gp120. J. Virol. **69:**101–109.
29. **Nitayaphan, S., J. A. Grant, G. J. Chang, and D. W. Trent.** 1990. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14–14–2. Virology **177:**541–552.
30. **Ogata, N., L. Ostberg, P. H. Ehrlich, D. C. Wong, R. H. Miller, and R. H. Purcell.** 1993. Markedly prolonged incubation period of hepatitis B in a chimpanzee passively immunized with a human monoclonal antibody to a determinant of hepatitis B surface antigen. Proc. Natl. Acad. Sci. USA **90:**3014–3018.
31. **Okuno, Y., T. Fukunaga, M. Tadano, Y. Okamoto, T. Ohnishi, and M. Takagi.** 1985. Rapid focus reduction neutralization test of Japanese encephalitis virus in microtiter system. Brief report. Arch. Virol. **86:**129–135.
32. **O'Leary, D. R., A. A. Marfin, S. P. Montgomery, A. M. Kipp, J. A. Lehman, B. J. Biggerstaff, V. L. Elko, P. D. Collins, J. E. Jones, and G. L. Campbell.** 2004. The epidemic of West Nile virus in the United States, 2002. Vector Borne Zoonotic Dis. **4:**61–70.
33. **Pech, M., H. Smola, H. D. Pohlenz, B. Straubinger, R. Gerl, and H. G. Zachau.** 1985. A large section of the gene locus encoding human immunoglobulin variable regions of the kappa type is duplicated. J. Mol. Biol. **183:**291–299.
34. **Persson, M. A., R. H. Caothien, and D. R. Burton.** 1991. Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. Proc. Natl. Acad. Sci. USA **88:**2432–2436.
35. **Rosen, L.** 1996. Dengue hemorrhagic fever. Bull. Soc. Pathol. Exot. **89:**91–93.
36. **Sabin, A. B.** 1952. Research on dengue during World War II. Am. J. Trop. Med. Hyg. **1:**30–50.
37. **Sanna, P. P., M. E. Samson, J. S. Moon, R. Rozenshteyn, A. De Logu, R. A. Williamson, and D. R. Burton.** 1999. pFab-CMV, a single vector system for the rapid conversion of recombinant Fabs into whole IgG1 antibodies. Immunotechnology **4:**185–188.
38. **Schofield, D. J., J. Glamann, S. U. Emerson, and R. H. Purcell.** 2000. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. J. Virol. **74:**5548–5555.
39. **Schofield, D. J., J. R. Stephenson, and N. J. Dimmock.** 1997. Variations in the neutralizing and haemagglutination-inhibiting activities of five influenza A virus-specific IgGs and their antibody fragments. J. Gen. Virol. **78:**2431–2439.
40. **Thullier, P., P. Lafaye, F. Megret, V. Deubel, A. Jouan, and J. C. Mazie.** 1999. A recombinant Fab neutralizes dengue virus in vitro. J. Biotechnol. **69:**183–190.
41. **Tomlinson, I. M., G. Water. J. D. Marks, M. B. Llewelyn, G. Winter.** 1992. The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J. Mol. Biol. **227:**776–798.
42. **Wang, W. K. D. Y. Chao, C. L. Kao, H. C. Wu, Y. C. Liu, C. M. Li, S. C. Lin, J. H. Huang, and C. C. King.** 2003. High levels of plasma dengue viral load during defervescence in patients with dengue haemorrhagic fever: implications for pathogenesis. Virology **305:**330–338.
43. **Wang, W. K. D. Y. Chao, S. R. Lin, C. C. King, and S. C. Chang.** 2003. Concurrent infection by two dengue virus serotypes among dengue patients in Taiwan. J. Microbiol. Immunol. Infect. **36:**89–95.
44. **Webster, R. G., J. A. Kasel, R. B. Couch, and W. G. Laver.** 1976. Influenza virus subunit vaccines. II. Immunogenicity and original antigenic sin in humans. J. Infect. Dis. **134:**48–58.
45. **Wood, C. R., A. J. Dorner, G. E. Morris, E. M. Alderman, D. Wilson, R. M. O'Hara, Jr., and R. J. Kaufman.** 1990. High level synthesis of immunoglobulins in Chinese hamster ovary cells. J. Immunol. **145:**3011–3016.
46. **Wu, T. T., G, Johnson, and E. A. Kabat.** 1993. Length distribution of CDRH3 in antibodies. Proteins **16:**1–7.