Development of Human-Murine Chimeric Immunoglobulin G for Use in the Serological Detection of Human Flavivirus and Alphavirus Antibodies

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Arthropod-borne viruses (arboviruses) are responsible for a number of medically important human diseases. These viruses are maintained in nature through biological transmission between susceptible vertebrate hosts by blood-feeding arthropods, primarily mosquitoes and ticks. Although over 150 arboviruses are known to cause disease in humans, the majority of medically important arboviruses are found in three separate families, the Flaviviridae, the Togaviridae (genus Alphavirus), and the Bunyaviridae (24). Transmission of arboviruses can vary by season, a consequence of the feeding patterns of their respective arthropod vectors, as well as by specific geographic location, as is seen for dengue fever virus (DENV) and Japanese encephalitis virus (JEV) (20, 24). The primary clinical manifestation of arboviral disease in North America is encephalitis, although some arboviruses, such as yellow fever virus (YFV) are capable of causing severe hemorrhagic disease as well. Prior to the 1999 outbreak of West Nile virus (WNV) encephalitis in New York City, St. Louis encephalitis virus (SLEV) was the most important agent of epidemic viral encephalitis in North America, last causing a major epidemic in the mid-1970s (26, 28, 33). Since 1999, the distribution of WNV has rapidly expanded from New York to the rest of the United States and into Canada, Central America, and South America. As of April 2009, a total of 29,598 human WNV cases in the United States had been reported to the Centers for Disease Control and Prevention, of which 1,159 resulted in death (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm).

Given the globalization of commerce and travel, virus-infected people, animals, and arthropod vectors are able to move more easily between locations with great speed (16). Thus, it is likely that other arboviruses will follow the example of WNV, resulting in new or novel disease outbreaks in regions of the world outside their normal geographic ranges. Therefore, a rapid and standardized approach to identification of arboviral infections is needed worldwide for the diagnosis and tracking of current and reemerging arboviral diseases.

In the past, identification of antiviral antibody relied on four tests: the hemagglutination inhibition test, the complement fixation test, the plaque reduction neutralization test, and the indirect fluorescent antibody (IFA) test. Positive identification of a viral infection required a 4-fold increase in titer between acute- and convalescent-phase serum samples in these assays (20). Rapid serologic assays, such as the IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) and IgG ELISA are now routinely used in diagnosis soon after infection. Early in infection, IgM antibody is more specific, while later in infection, IgG antibody is more cross-reactive. Inclusion of murine monoclonal antibodies (mMAbs) with defined virus specificities in these solid-phase assays has permitted a level of assay standardization that was not previously possible (30). In the diagnostic laboratory, the MAC-ELISA and the IgG ELISA are often used in tandem to identify positive specimens based on a 4-fold increase in titer between acute- and convalescent-phase serum samples and have replaced the more time-consuming and labor-intensive assays (11, 16, 21).

Application of the ELISA in serodiagnosis of arboviral infection is most hampered by the limited availability of human infection-immune sera for use as virus-reactive, antibody-pos-
itive control specimens. For the most part, antibody-positive control sera are derived by pooling small volumes of antibody-positive diagnostic serum specimens. The specimens are typically obtained for only the most prevalent arboviral agents (20, 21). Lot-to-lot variability of these serum pools can be high, and constant recollection and recalibration of antibody-positive and -negative control sera are necessary to ensure that test parameters remain valid (10, 21). Of even greater concern is the lack of antibody-positive control sera that can be used in diagnostic ELISAs to identify arboviruses that currently cause rare or infrequent human infections (20).

The replacement of variably reactive human control sera with group-specific human IgG antibodies would be a tremendous asset in the serological diagnosis of arboviral infections. Although a number of mMAbs demonstrating flaviviral, alphaviral, or bunyaviral group reactivity exist, they are unsuitable for use as positive serum controls in ELISAs designed to detect the presence of human antibodies. Moreover, the capture or detector antibodies used in these assays are often designed to react with other murine components of the ELISA, leading to an overwhelming false-positive response if mMAbs are employed as positive controls.

Fortunately, advances in the humanization of mMAbs have made it possible to overcome these limitations (31). One such method involves the incorporation of the heavy (H)- and light (L)-chain variable (V) regions of a given mMAb into an expression plasmid containing the constant (C) region of human IgM (10). Upon transfection of cells, the resulting plasmid construct expresses a human-murine hybrid IgM chimeric mAb (cMAb) molecule that retains the specificity of the “parent” mMAb but reacts like human IgM in the MAC-ELISA (10, 12, 32).

We have previously reported on the construction and evaluation of an IgM cMAb with the specificity of the broadly flavivirus cross-reactive mMAb 6B6C-1. The 6ME2 IgM cMAb reacted with each flaviviral suckling mouse brain (SMB) or virus-like particle (VLP) antigen tested in the MAC-ELISA and displayed a strong preference for the WNV VLP antigen. The use of cell culture viral seed in place of the SMB or VLP antigens in the MAC-ELISA format resulted in enhanced reactivity, as measured by the maximum dilution of cMAb yielding a positive P/N value (positive/negative ratio; see below for details) against WNV, SLEV, DENV serotype 2 (DENV-2), and YFV. In this report we describe the development and characterization of two new IgG cMAbs for use in the indirect IgG ELISA. These cMAbs were created by incorporating the V regions of 6B6C-1 or the alphavirus group-specific mMAb 1A4B-6 into a plasmid construct containing the human IgG γ1 chain. The alpha- or flaviviral group reactivity of each cMAb was confirmed and subsequently evaluated in the standard indirect IgG ELISA. The cMAb demonstrating alphaviral (1GD5) or flaviviral (6GF4) group reactivities were selected for further use and were satisfactory replacements for anti-body-positive human control sera against all alphaviruses or flaviviruses tested.

**MATERIALS AND METHODS**

**Cell lines.** The Sp2/0-Ag14 (Sp2) murine myeloma, 6B6C-1 murine hybridoma, and 1A4B-6 murine hybridoma cell lines have been previously described (9, 12, 14, 25, 27, 32). Cells were propagated in hybridoma growth medium (HGM [high-glucose Dulbecco minimal essential medium containing L-glutamine supplemented with 20% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.15% sodium bicarbonate, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.1 mM nonessential amino acids]) unless noted otherwise. Cell culture viral seeds for WNV, YFV, JEV, SLEV, and DENV-2 were prepared as previously described (32).

**Isolation of immunoglobulin V regions.** A Pharmacia QuickPrep mRNA purification kit (Amersham Pharmacia, Piscataway, NJ) was used according to the manufacturer’s specifications to isolate mRNA from 5 × 10^7 hybridoma cells. PCR cloning of mMAb 6B6C-1 and 1A4B-6 V regions was performed as previously described (10, 32) using the first-round PCR primers listed in Table 1. For heavy-chain 3′ primers, M-IgG2a was used for mMAb 6B6C-1 and M-IgG2b was used for mMAb 1A4B-6. Primers were provided by the CDC Biotechnology Core Facility (Atlanta, GA). PCR-derived products were isolated by the Qiagen purification kit (Qiagen Inc., Valencia, CA), cloned into plasmid pCR2.1-TOPO (Invitrogen, Baltimore, MD), and subsequently used to chemically transform TOP10 competent Escherichia coli (Invitrogen) in accordance with the manufacturer’s protocol.

**Modification of V regions.** Variable light (kappa) (V_{\kappa}) and heavy (V_{\lambda}) regions of mMAbs 6B6C-1 and 1A4B-6 were further modified by a second round of PCR (Table 1) in order to add partial 5′ leader sequences, 3′ splice donor junctions,
antigens (VLP or SMB) diluted in PBS-T were added to each well (50 μl/H9262) and incubated overnight at 4°C. Alternatively, flaviviral cell culture seeds were used. Briefly, Immulon II HB flat-bottomed 96-well plates were coated at 4°C overnight with Flavi- or alphaviral protein antigens (normal SMB or COS-1 antigens) were obtained from the CDC Diagnostic branch. Culture supernatants of WNV (NY99), DENV-2 (30 PA), SLEV (strain TBH-2), YFV (strain 17D), DENV2 (30 PA), and cell culture supernatants containing IgG cMAbs were serially diluted 2-fold in PBS-T before being dispensed into appropriate wells; in some cases, purified cMab was diluted 1:10 or 1:100 in PBS-T prior to serial dilution in order to better visualize titration curves. Secondary antibody consisting of alkaline phosphatase-conjugated goat anti-human IgG (heavy chain; Jackson Immunoresearch) diluted 1,500,000 in PBS-T was added to each well (50 μl/well) and allowed to incubate for 1 h at 37°C. Substrate (Sigma Fast p-nitrophenyl phosphate tablet sets) was added to each well (75 μl/well) and allowed to incubate for 30 to 60 min at 25°C. Absorbance values at 405 nm (A405) were read using an ELISA808 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT). All ELISAs were performed in triplicate.

Test validation and calculation of P/N values. Test validation and positive-to-negative ratio (P/N) values were determined according to the procedure of Martin et al. (21) using internal positive and negative serum controls. The N value for each viral antigen was defined as the average A405 neg, normal human serum reacted with a given viral antigen. The P value of 6GF4 or 1GD5 IgM cMab for each viral antigen was determined to be the average A405 pos, positiveserum in the flavivirus MAC-ELISA (32). For 1A4B-6, the heavy- and kappa-chain variable regions for 1A4B-6 were submitted to GenBank and assigned the indicated accession numbers: 1A4B-6 VH, accession number GU724332; 1A4B-6 VK, accession number GU724341.

RESULTS

Cloning and sequencing of mMAb 1A4B-6 variable regions. In order to generate alphavirus or flavivirus group-specific IgG cMAbs, hybridomas of mMAbs with group-specific activities for flaviviruses (6B6C-1) or alphaviruses (1A4B-6) were grown in cell culture. Both 6B6C-1 and 1A4B-6 were used regularly by the CDC DRL for ELISA-based serodiagnosis of arboviral infection (15, 16, 21, 26). The cloning and sequencing of variable regions from the 6B6C-1 mMAb have been previously documented in a related study involving the development of a flavivirus group-specific human-murine chimeric IgM for use in the flavivirus MAC-ELISA (32). For 1A4B-6, the heavy- and kappa-chain variable regions (VH and VK, respectively) were cloned by reverse transcription-PCR using a combination of degenerate primers that annealed to conserved VH and VK gene leader sequences and C region-specific primers (10). Multiple clones of each V gene product were sequenced to ensure against possible DNA polymerase-induced errors. The 1A4B-6 VH and VK cDNA were sequenced, and a consensus of multiple sequence determinations was derived (Fig. 1a and b, respectively).

Assembly of the 6B6C-1 and 1A4B-6 IgG cMAb plasmid constructs (pdHL2-6G or pdHL2-1G). The 6B6C-1 and 1A4B-6 V regions were further modified by a second round of PCR to prepare VV and VH cDNA for insertion into the human IgG expression plasmid pdHL2. Primers used in this second-round PCR were designed to incorporate 3′ splicing do-
nor junctions in order to ensure correct expression of the
6B6C-1 and 1A4B-6 murine V regions with the human C
region functional splice acceptor sites located in pdHL2. The
second-round PCR primers also added appropriate restriction
endonuclease sites at either end of each V region to permit
subsequent cloning of the finished V<sub>K</sub> and V<sub>H</sub> inserts into
pdHL2. The pdHL2 plasmid contains genomic clones of the
human kappa (C<sub>K</sub>/H9010) and IgG (C<sub>H</sub>/H9253) C region genes, both of
which are controlled by a metallothionein I promoter and a
mouse immunoglobulin H chain enhancer. Plasmid pdHL2
also contains an altered dihydrofolate reductase gene that al-
lows for selective growth in media containing methotrexate.
The 1A4B-6 V regions were cloned into pdHL2, forming plas-
mid pdHL2-1G; the 6B6C-1 V regions were cloned into
pdHL2, forming plasmid pdHL2-6G.

Expression and purification of cMAbs. Cell-free superna-
tants of Sp2 cells transfected with either pdHL2-1G or
pdHL2-6G were analyzed by ELISA for the presence of
cMAbs approximately 2 weeks following transfection. A total
of 7 separate wells (out of 90) containing Sp2 cells transfected
with plasmid pdHL2-1G tested positive for human IgG when evaluated
by ELISA; of those transfected with plasmid pdHL2-6G, 2 of
90 were positive for human IgG. These nine transfectants were
next tested for specific antiflaviviral or antialphaviral activity by
indirect IgG ELISA using either SLEV or EEEV SMB anti-
gens, respectively. Of the seven pdHL2-1G transfectants, five
demonstrated specific antialphaviral activity; both pdHL2-6G
transfectants were positive for antiflaviviral activity. Multiple
passages of those transfectants demonstrating antiflaviviral or
antialphaviral activity were analyzed by indirect IgG ELISA to
ensure that plasmid retention over repeated cell culture pas-
sage was maintained and also allowed for the selection of those
cells that consistently produced the highest antiflaviviral (6GF4
IgG) or antialphaviral (1GD5 IgG) activity as measured by
ELISA. Sp2 cells secreting 6GF4 or 1GD5 were sent to QED
Bioscience, Inc., for further purification. Purified lots of each
human-murine chimeric antibody were received from QED
Bioscience and subsequently evaluated for activity against mul-
tiple alphaviruses or flaviviruses by indirect IgG ELISA.

Arboviral group reactivity of cMAbs. The 6GF4 and 1GD5
cMAbs were assayed for specific group reactivity by indirect
IgG ELISA using SMB or VLP antigens of prominent mem-
bers of the family Flaviviridae or Alphaviridae, respectively. The
6GF4 IgG, at a dilution of 1/125, reacted positively (P/N
FIG. 1. Nucleotide and deduced amino acid sequences of the pdHL2-1G V regions. +1, denotes start of mature protein; solid-line box, CDR
1; dotted-line box, CDR 2; dashed-line box, CDR 3. (a) pdHL2-1G V<sub>K</sub> region derived from 1A4B-6; (b) pdHL2-1G V<sub>H</sub> region derived from
1A4B-6.
value of >3.0) with all flaviviral antigens tested, with the exception of the SLE SMB antigen, which required a higher concentration of cMAB (1:160 dilution) to achieve a P/N value greater than 3.0 (Table 2). The 6GF4 IgG reacted exceptionally well with those antigens demonstrating high Pmax/N values: YFV (SMB), WNv (VLP), and JEV (VLP). Against these antigens, the 6GF4 IgG, at dilutions of ≥1:25,600, gave P/N values of >3.0. When flaviviral VLP or SMB antigens were replaced with cell culture viral seed in the indirect IgG ELISA, Pmax/N values of 6GF4 IgG for each flavivirus changed to the following: for WNv, 3.50; DENV-2, 3.14; YFV, 3.33; SLEV, 4.65; JEV, 5.87. Cell culture viral seed for POWV was unavailable at the time of testing.

For the alphavirus group-specific 1GD5 IgG, dilutions of 1:64 or greater were able to yield positive ELISA reactions (P/N > 3.0) with all alphaviral antigens assayed (Table 3). Although the 1GD5 IgG produced the highest overall Pmax/N values against the WEEV and VEEV SMB antigens, the 1GD5 IgG also reacted very well with the EEEV SMB antigen (P/N, >3 against viral antigens) than in that demonstrated by the 6GF4 IgG with flaviviral antigens, which showed a broad range of activity.

Samples of human infection-immune-positive control serum were included in each indirect IgG ELISA performed, and the resulting Pmax/N values were compared to the Pmax/N values obtained with the 6GF4 or 1GD5 IgG cMABs. For the most part, the Pmax/N values produced by the chimeric IgG antibodies were comparable to those obtained using positive human control sera at previously determined optimum dilutions (Table 2 and Table 3). In the case of YFV and DENV-2, the positive human sera were unable to achieve Pmax/N values greater than 3.0; a sample of human serum positive for WEEV was unavailable at the time of these experiments. Absorbance values of purified chimeric IgG (at maximum dilutions yielding P/N values of >3 against viral antigens) against normal mouse brain antigen or normal COS antigens were at least 3-fold lower than those measured against viral antigens at identical antibody dilutions.

**DISCUSSION**

Antibody-positive human control sera for use in the MAC and indirect IgG ELISA are derived from small volumes of serum specimens submitted to the CDC for diagnostic purposes. Not surprisingly, these specimens are typically collected only from the most prevalent arboviral infections, thus limiting the application of ELISA in arboviral surveillance to the narrow range of diseases currently being diagnosed or tracked by disease surveillance systems at any given time. Furthermore, positive control serum pools prepared from these specimens suffer high lot-to-lot variability that necessitates constant recalibration of serological potency and coverage. Of even greater concern is the lack of antibody-positive control sera that can be used in these ELISAs for the identification of infrequent or emerging arboviruses (20, 21). In this study, we have demonstrated that IgG cMABs expressing the variable region specificity of the mMABs 6B6C-1 (eMAB 6GF4) or 1A4B-6 (cMAB 1GD5) can serve as suitable replacements for human control sera in the indirect IgG ELISA used in the serodiagnosis of arboviral disease. These cMABs offer diagnostic laboratories an unlimited supply of control reagents of a set affinity and specificity, in known quantities that should facilitate diagnostic testing and lab-to-lab comparative evaluations.

A number of techniques have recently been described for engineering human antibodies. Transgenic mouse strains carrying human heavy- and light-chain loci, the immortalization of human B cells through viral transformation, and production of human hybridomas using new human fusion partner cell lines are all methods capable of producing human monoclonal antibodies (6, 18, 24). Unfortunately, these methods do not facilitate the design of human MAbs of a defined specificity. A considerable amount of additional screening would be required to identify specific group-reactive antibodies. An alternative to producing fully human MAbs is humanizing existing murine MAbs of known specificity. The flavivirus group-specific 6B6C-1 mMAB was originally raised against SLEV and is specific for the flaviviral envelope (E) protein (22, 27); mMAB 1A4B-6 reacts specifically with the E1d domain of the EEEV E1 glycoprotein and is alphavirus group reactive (14, 25). Both 6B6C-1 and 1A4B-6 are regularly used in serological assays as capture antibodies and antibody-enzyme conjugate detectors and were likely candidates for humanization (15, 16, 20, 21, 23, 28, 29). Using the pdHL2 IgG expression vector along with RNA purified from the 6B6C-1 and 1A4B-6 hybridomas, we prepared IgG eMAbs 6GF4 and 1GD5 for use in the indirect IgG ELISA.

The 6GF4 IgG cMAB was able to achieve positive P/N values with each flaviviral antigen tested in the indirect IgG ELISA. 6GF4 demonstrated a strong preference for the YFV, WNv, and JEV antigens compared to the relatively weaker reactions with DENV-2, POWV, or SLEV antigens. The 1GD5 IgG cMAB similarly reacted positively with all alphavi-
ruses tested, and there appeared to be much less variability in the reactivity of 1GD5 when assayed against the representative members of the alphavirus family.

Although it was somewhat surprising to see that the 6GF4 cMAb reacted weakly with SLEV, the virus initially used to generate the 6B6C-1 mMAb, it is not all that alarming considering that the reactivity and quality of SMB and VLP antigens can vary greatly from one lot of antigen to the next. Furthermore, when tested against viral seed antigens instead of the SMB or VLP antigens in the indirect IgG ELISA format, the 6GF4 cMAb reacted better with the SLEV seed antigen, generating the second-highest overall FmaN value (4.65) of all flavivirus viral seed antigens tested. The 6ME2 IgM cMAb described in a previous publication also demonstrated a relatively low reactivity with the SLEV SMB antigen and reacted strongly with the SLE viral seed antigen (32). This disparity between the viral SMB antigen and the viral seed antigen might be attributed to differences inherent in the SLEV strains used and/or differences in the preparation of each antigen; the SLEV SMB antigen used in both the IgG and IgM ELISA studies was prepared from strain TBH-28, an isolate taken postmortem from cases of fatal SLE in the Tampa Bay area of Florida in 1962, while the viral seed antigen was prepared from Vero cells infected with SLEV strain MSI-1, the same SLE strain that was originally used to generate the 6B6C-1 mMAb. However, given the homogeneity of the 6B6C-1 epitope among all flaviviruses (5), the variable reactivity of 6GF4 with different flaviviral antigens is most likely due to the quality and concentration of the specific antigen lots available from the CDC DRL used in this assay rather than the preference of the chimeric antibody for a specific flavivirus.

The viral antigens included in these ELISAs are representative of the major flavivirus or alphavirus antigen complexes with members causing human disease. Therefore, it is reasonable to assume that these chimeric antibodies would be useful as positive controls in the serodiagnosis of all flaviviral or alphaviral specimens. One possible complication with using a MAAb-derived chimeric antibody as a positive control reagent would be the occurrence of an arbovirus with an altered E glycoprotein (flavivirus) or E1 glycoprotein (alphavirus) rendering it nonreactive with the group-specific activities of 6B6C-1 or 1A4B-6. The epitope defined by 6B6C-1 and other E protein-specific flavivirus group-reactive mMAbs has recently been mapped to the E protein fusion loop (5). This sequence is highly conserved among all flaviviruses, probably because of its critical interaction with cell membranes during virus replication. The alphavirus E1 glycoprotein is also reported to be responsible for membrane fusion, and although no epitope map yet exists for the alphavirus E1 glycoprotein, the crystal structure of E1 has been determined and was found to share significant homology and identical topology with the flavivirus E glycoprotein (7, 8). Furthermore, we have shown that a flavivirus transmitted by a vector other than the mosquito, POWV, reacts with the 6GF4 IgG cMAb. This finding agrees with those previously reported by the CDC DRL that both tick-borne encephalitis virus and POWV react with the 6B6C-1 mMAb in the MAC-ELISA format (32).

Given the respective group reactivities of 6B6C-1 and 1A4B-6, the discovery of a nonreactive flavivirus or alphavirus is unlikely. If such a virus was isolated, however, one solution would be to develop a combination of two different chimeric antibodies sharing group reactivity but for separate epitopes. Additionally, since the current indirect IgG-ELISA formats utilize a 6B6C-1–enzyme conjugate detector in flavivirus serodiagnosis and a 1A4B-6 capture antibody in alphaviral serodiagnosis, any arbovirus expressing a modified or nonreactive glycoprotein would not likely be detected by the currently employed assays.

The incorporation of cMAbs in immunoassays that rely on variably reactive human sera as controls will provide diagnostic laboratories with an unlimited supply of control reagents of a set affinity and specificity. Also, the use of a positive control with the specificity of a MAAb allows for better characterization of unknown specimens than that of polyclonal sera with heterogeneous reactivities. The 6GF4 and 1GD5 IgG cMAbs should offer viable alternatives to positive human serum controls for flavivirus or alphavirus detection via the indirect IgG ELISA. Together with the previously described 6ME2 IgM cMAb, we now possess positive controls capable of evaluating both acute- and convalescent-phase sera for possible flavivirus infection. An IgM cMAb with alphavirus group specificity has also been developed, is currently being characterized for utilization in the MAC-ELISA for serodiagnosis of alphaviral disease, and will complete the set of cMAbs necessary to identify both classes of human antibodies developed in a wide variety of human arboviral infections.

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