Antibody Responses Are Generated to Immunodominant ELK/KLE-Type Motifs on the Nonstructural-1 Glycoprotein during Live Dengue Virus Infections in Mice and Humans: Implications for Diagnosis, Pathogenesis, and Vaccine Design

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Received 7 October 2006/Returned for modification 12 December 2006/Accepted 16 February 2007

Antibodies generated to the purified dengue type 2 virus (D-2V) nonstructural-1 (NS1) protein in mice and rabbits were compared with those generated to this protein in congenic (H-2 class II) mouse strains and humans after D-2V infections. Unlike the profiles observed with the rabbits, similar antibody reaction profiles were generated by mice and humans with severe D-2V disease (dengue hemorrhagic fever [DHF]/dengue shock syndrome [DSS]). Many of these epitopes contained the core acidic-hydrophobic-basic (tri-amine-acid; ELK-type) motifs present in the positive or negative orientations. Antibody responses generated to these ELK/KLE-type motifs and the epitope LX1 on this protein were influenced by class II molecules in mice during D-2V infections; but these antibodies cross-reacted with human fibrinogen and platelets, as implicated in DHF/DSS pathogenesis. The core LX1 epitope (113YWKTIG119), identified by the dengue virus complex-specific monoclonal antibody (MAb) 3D1.4, was prepared so that it contained natural I-A\(^{\beta}\)-binding and ELK-type motifs. This AFLX1 peptide, which appropriately displayed the ELK-type and LX1 epitopes in solid-phase immunoassays, generated a similar, but lower, immunodominant anti-ELK-motif antibody reaction in I-A\(^{\beta}\)-positive mice, as generated in mice and humans during D-2V infections. These antibody responses were much stronger in the high-responding mouse strains and each of the DHF/DSS patients tested and may therefore account for the association of DHF/DSS resistance or susceptibility with particular class II molecules and autoantibodies, antibody-stimulating cytokines (e.g., interleukin-6), and complement product C3a being implicated in DHF/DSS pathogenesis. These results are likely to be important for the design of a safe vaccine against this viral disease and showed the AFLX1 peptide and MAb 3D1.4 to be valuable diagnostic reagents.

The four serotypes of dengue viruses have spread throughout the tropical and subtropical belts of the world, resulting in a globally increased incidence of the severe dengue viral disease dengue hemorrhagic fever (DHF; grades I to IV) (14). Cases of DHF are discriminated from cases of classical dengue fever (DF), in which hemorrhage may also occur, by evidence of vascular leakage (hemoconcentration) (29), where DHF grades III and IV (dengue shock syndrome [DSS]) are characterized by narrowed pulse pressures (hypotension) and undetectable pulse pressures (profound shock), respectively (29). Sequential infections with virulent strains of each dengue virus serotype have been implicated in the pathogenesis of DHF/DSS (15). The correlation of disease severity with the levels of markers of immune activation (e.g., interleukin-6 [IL-6], IL-8, tumor necrosis factor alpha, gamma interferon, and the soluble tumor necrosis factor alpha receptor [p75]), together with altered platelet, dendritic cell, monocyte, and T-cell functions (12, 13, 22), strongly implicates inappropriate immune activation in the pathogenesis of DHF/DSS. Clinically graded dengue viral disease severity has also been found to strongly correlate with reductions in platelets and fibrinogen concentrations, with increased concentrations of vasoactive histamine and complement product C3a, and with the localization of antibodies, complement, and fibrinogen on the vascular endothelia of DHF/DSS patients (2). These results therefore strongly implicate autoantibody reactions in the pathogenesis of DHF/DSS. To account for these findings, a mouse monoclonal antibody (MAb), MAb 1G5.4-A1-C3, which reacted with the nonstructural-1 (NS1) proteins of each dengue virus serotype but none of the other flaviviruses tested (6), defined multiple acidic (E or D)-aliphatic/aromatic (G, A, I, L, or V/F, W, or Y)-basic (K or R) (tri-amine-acid) (ELK-type) motifs present in either orientation (ELK/KLE-type motifs) in linear (sequential) epitopes and functional sites (e.g., RGD motifs) on human blood proteins (e.g., fibrinogen) and integrin/adhesion molecules, such as \(\alpha\)IIb on platelets, ICAM-1 on endothelial cells, and \(\beta3\) on both platelets (\(\alpha\)IIb\(\beta3\)) and endothelial cells (\(\alpha\)V\(\beta3\)) (6). Mice immunized with the dengue type 2 virus (D-2V) NS1 protein generated polyclonal antibodies (PAb8) which showed similar anti-ELK/KLE-type motif specificities as MAb 1G5.4-A1-C3 against a set of 174 synthetic peptides sequentially spanning the D-2V NS1 protein and also cross-reacted with human fibrinogen, endothelial cells, and platelets (6). The autoantibodies generated to these ELK/KLE-type motifs during human dengue virus infections were therefore hypothesized to form circulating immune complexes with human blood-clotting proteins and to cause pathological effects on human platelets and endothelial...
cells which could account for the thrombocytopenia and vascular leakage observed during DHF/DSS (6). Cross-reactive antibodies to fibrinogen (and plasminogen) generated in human DHF/DSS patients could not, however, be detected in immunoassays due to cross-reaction of the labeled secondary antibody with this protein and because they were thought to rapidly fix complement in vivo (6, 7). Their reactions were, instead, confirmed by identifying immunoglobulin M (IgM) and IgG together with the complement proteins C1q and C3 and high concentrations of fibrinogen as well as lower concentrations of plasminogen, but no dengue virus proteins, in DSS patients’ high-molecular-weight circulating immune complexes (7). More recently, higher concentrations of IgM and IgG were found on the surface of platelets from DHF/DSS patients than on those from DF patients (26), and DHF/DSS patients’ antibodies were shown to cross-react with human endothelial cells (21). The role of these ELK/KLE-type motifs in the pathogenesis of DHF/DSS, however, still needs to be confirmed by comparing the PAb reactions of DF and DHF/DSS patients against the epitopes defined by MAb 1G5.4-A1-C3 and mouse PAbs generated to the D-2V NS1 protein, as performed in other studies of microbial molecular mimicry implicated in human autoimmune diseases (23).

Four other MAbs were shown to define the same 9- to 11-amino-acid sequence (epitope LX1) on the NS1 proteins of each dengue virus serotype by using sets of synthetic peptides (11), and these findings were further supported by the results from competition studies (P. R. Young, personal communication). Although the average immunoblot reaction intensities of these four MAbs with the NS1 proteins of each dengue virus serotype and other flaviviruses were shown for brevity (10), one of them showed a different anti-NS1 protein reaction profile within the dengue virus antigenic complex and only some of them weakly cross-reacted with the NS1 proteins of representatives of flavivirus antigenic complex III (e.g., Japanese encephalitis [JE] virus), while none of them reacted with the NS1 proteins of any other flaviviruses than those described previously (6, 10, 11). The mapping of epitopes on the NS1 proteins with 174 overlapping nonapeptide sequences spanning the entire D-2V NS1 glycoprotein was described previously (9). Briefly, the clarified supernatants from D-2V (strain TR1751)-infected Vero cells were adjusted to 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA (TNE buffer) containing 0.02% (wt/vol) NaN3 and a mixture of protease inhibitors (Sigma). These supernatants were then passed through an immunoaffinity column containing MAb 3D1.4. After the mixture was washed with TNE buffer containing protease inhibitors, the bound D-2V NS1 protein was eluted with TNE buffer containing 20 mM diethylamine (pH 11.2), and 0.5-mL fractions were immediately neutralized with 0.1 mL of 1 M Tris-HCl (pH 7.2). The concentration of the D-2V NS1 protein in each fraction was determined by a biocinchonic acid protein assay (Pierce) with standard bovine serum albumin concentrations.

Production of MAbs and PAbs in mice and rabbits. The generation of mouse and rabbit PAbs and the production of mouse MAbs were performed under a mouse personal animal procedures license (PIL 70/6903) issued by the Home Office of the United Kingdom. Blood samples from the retroorbital sinus were obtained from mice by using sterile fine-bore Pasteur pipettes after anesthesia with 3% halothane (Rhone Merieux, Ireland) in oxygen at 1 dm3/min. The production of MAbs and PAbs to the D-2V NS1 protein and their immunoblot reactions with the NS1 proteins of the dengue viruses and other flaviviruses were described previously (6, 10, 11). The mapping of epitopes LD2, 24A, LX1, 24B, and 24C and multiple ELK/KLE-type epitopes on the dengue virus NS1 proteins with 174 overlapping nonapeptide sequences spanning the entire D-2V NS1 protein sequence and peptides containing the corresponding sequences from the NS1 proteins of the other dengue virus serotypes was also described (6, 7, 10, 11). The production of PAbs in outbred Tyler’s isogenic and congenic (H-2 class II) mouse strains and outbred rabbits (New Zealand White) to the purified D-2V NS1 protein was described previously (6, 10). Briefly, outbred Tyler’s original or congenic (B10.G, I-Ak; B10.RIII, I-Ar, I-Er; B10.M, I-Af; B10.S, I-As; C57BL/6J, I-Ak; B10.BR, I-Ab; B10.A, I-Ak; and B10.DN2: I-Ak, I-Ea) mouse strains (Harlan-Olac, United Kingdom) were immunized by a combination of the subcutaneous (i.c.) and the intraperitoneal (i.p.) routes with 10 μg of the purified dimeric D-2V (strain TR1751) NS1 protein emulsified in Freund’s complete adjuvant and were boosted 2 weeks later by the same routes and with the same antigen dose contained in phosphate-buffered saline (PBS). Blood samples were obtained from the retroorbital sinus 2 weeks later, and the sera were stored at −80°C. New Zealand White rabbits were immunized s.c. at multiple sites with a total of 50 μg of the D-2V NS1 protein emulsified in Freund’s incomplete adjuvant. Three weeks later, they were boosted by the same route with the same antigen dose emulsified in Freund’s incomplete adjuvant. A final immunization with the same antigen dose in PBS FALCONAR CLIN. VACCINE IMMUNOL. 504

MATERIALS AND METHODS

Flavivirus growth. The growth of flaviviruses in mammalian fibroblast (Vero) cells was described previously (6, 10, 11). Briefly, representative viruses from flavivirus antigenic complex I (tick-borne encephalitis [TBE; Neudorf] virus), complex III (JE [strain Nakayama], WN [strain E101], St. Louis encephalitis [SLE; strain 947-1] viruses), complex VII (dengue type 1 virus [D-1V; strain Hawaiian 1944], D-2V [strain TR1751], dengue type 3 virus [D-3V; strain H87], and dengue type 4 virus [D-4V; strain Dominica]), and complex U (unassigned) (YF [strain Asibi] virus) were used to infect 70% confluent Vero cell monolayers maintained in medium 199 (DCCM M 6690; Sigma) containing 0.18% (wt/vol) NaHCO3, 3.5% (vol/vol) fetal bovine serum, and antibiotics (growth medium). After incubation at 37°C for 4 or 5 days, the supernatants were harvested and replaced with fresh virus growth medium, and the fluids were incubated for a further 3 to 4 days, when a second supernantant was harvested. These supernatants were pooled, clarified by centrifugation, and then stored at −80°C. The virus-infected cell monolayers were homogenized with 0.5% (wt/vol) sodium dodecyl sulfate (SDS) containing 330 mM phosphoric acid and 0.71% (wt/vol) Trisma base (pH 6.8) (0.1 mL/cm2 of cell monolayer) (cell lysis buffer), before storage at −40°C. Titers of the live D-2V stocks used to infect the mice were determined by plaque assays with Vero cells, as described previously (5). Purification of the D-2V NS1 protein. The purification of the native, dimeric form of the D-2V NS1 glycoprotein was described previously (9). Briefly, the clarified supernatants from D-2V (strain TR1751)-infected Vero cells were adjusted to 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5 mM EDTA (TNE buffer) containing 0.02% (wt/vol) Na2SO4, and a mixture of protease inhibitors (Sigma). These supernatants were then passed through an immunoaffinity column containing MAb 3D1.4. After the mixture was washed with TNE buffer containing protease inhibitors, the bound D-2V NS1 protein was eluted with TNE buffer containing 20 mM diethylamine (pH 11.2), and 0.5-mL fractions were immediately neutralized with 0.1 mL of 1 M Tris-HCl (pH 7.2). The concentration of the D-2V NS1 protein in each fraction was determined by a bicinechonic acid protein assay (Pierce) with standard bovine serum albumin concentrations.

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was given 3 weeks later by the intramuscular route, 30 to 40 ml of blood was obtained from their marginal ear veins 2 weeks later, and the sera were stored at −80°C.

In this study, 3-week-old mice of the same congeneric mouse strains (see above) (three mice/strain) were infected with 3.2 \times 10^6 PFU of D-2V (strain TR1751) contained in 0.5 ml of virus growth medium by the combined s.c. and i.p. routes and were boosted with the same dose of live D-2V by the same routes 2 weeks later. Blood samples were collected 2 weeks after the first and second infections, and the sera were stored at −80°C.

**Serum samples from dengue virus-infected patients.** Paired serum samples from patients classified with DF or DSS by using the WHO guidelines (29) were provided by S. K. Lam from the WHO Virus Reference Laboratory, University of Malaya, Kuala Lumpur, Malaysia. Acute secondary dengue virus infections were confirmed by observing high dengue virus-specific IgG antibody titers in these patients’ acute-phase serum samples that increased by greater than four-fold in their convalescent-phase serum samples, collected 2 to 14 days later, as determined by using an IgG-capture enzyme-linked immunosorbent assay (ELISA), as described previously (8). D-2V infections were confirmed by virus isolation in C3/32 cell culture and subsequent serotype identification with dengue virus serotype-specific MABS, as described previously (8).

**Immunosassay.** The optimization and use of the indirect ELISAs with the purified D-2V NS1 protein, human fibrinogen, human platelets, human serum albumin, and chicken egg albumin were described previously (6, 7, 10, 11). For these assays, ELISA plates (Immulon 2; Dynatech, United Kingdom) were coated at 10 μg/ml (50 μl/well) with the purified antigens in sodium carbonate/bicarbonate buffer (pH 9.6). After the plates were washed with PBS, they were blocked with 1% (wt/vol) gelatin in PBS. Serial three- to four-fold dilutions of the mouse MABS or mouse, rabbit, or human PABS were prepared in PBS containing 0.02% (vol/vol) Tween 20 (PBS/T; P 1379; Sigma) with 0.25% (wt/vol) gelatin (PBS/T/G), and the plates were incubated at 25°C for 2 h. These plates were then washed with PBS/T; a 1/1,000 dilution of peroxidase-labeled goat anti-human (110-035-088), anti-mouse (115-035-062), or anti-rabbit (111-035-144) IgG (heavy and light chains; Jackson ImmunoResearch) in PBS/T/G was added; and the mixture was incubated at 25°C for 2 h. After the plates were washed with PBS/T, bound antibodies were detected by the addition of 0.14% (vol/vol) o-phenylenediamine dihydrochloride (P 1526; Sigma) with 0.003% (vol/vol) H_2O_2 in citrate/phosphate buffer (pH 5.0) (50 μl/well), the reaction was stopped with 0.2 M H_2SO_4 (25 μl/well), and the absorbance values were recorded at dual wavelengths of 490 nm and 630 nm (MRX; Dynex).

To purify human platelets, venous blood from a healthy human was collected in 2 ml of sodium ethylenediaminetetraacetic acid and centrifuged at 200 × g for 20 min at 25°C, and the platelets were collected from the upper layer. These cells were washed four times in 0.34% (wt/vol) sodium ethylenediamine-tetraacetic acid in PBS (pH 7.2) by centrifugation at 1,000 × g for 20 min at 25°C, and the platelets were collected from the upper layer of the tube. These sera were initially tested. Pools of PABS rather than individual PABS generated in congeneric mice were also tested against these peptide-coated pAbs/serum since the individual serum samples from each group of congeneric mice showed only minor variations either in their ELISA titers against the purified D-2V NS1 protein or in their immunoblot reaction profiles against the NS1 proteins of each dengue virus serotype (6). The reactions of these PABS pools against the LX1 ELK-type peptides were then compared to those obtained with individual serum samples from panels of patients with DF or DSS.

The AFLX1 peptide (amino acids 110 to 129 of the D-2V [strain PR159S1 and TR1751] NS1 protein) was altered from that described previously (6) by the addition of the natural carboxyl-terminal histidine residue. This peptide was prepared at a 20 μM scale on 200- to 400-mesh Fmoc-cysteine (trityl) Wang resin by using Fmoc amino acids activated with o-benzoctriazol-1-yl-N,N,N’,N”-tetramethyluronium hexafluorophosphate/diisopropylthlamine and coupled at an eightfold excess. The peptide was then cleaved by using 2% (vol/vol) phenol, 1% (vol/vol) 1.2-ethanediethiol, 2% (vol/vol) H_2O_2, 1.5% (vol/vol) acetic acid, and 1.2% (vol/vol) triisobutylamine in trifluoroacetic acid (Fluka, Switzerland). The cleaved peptide was then repeatedly washed in cold peroxide-free diethyl ether (20 × g for 20 min) and finally dried under argon gas. After resuspension in 5 ml of 0.1% (vol/vol) acetonitrile in H_2O, this peptide was purified on a preparative C_18 reverse-phase column (Vydac) by using a 5 to 95% (vol/vol) acetonitrile-H_2O gradient containing 0.1% (vol/vol) trifluoroacetic acid, the main peak was detected at 215 nm (Beckman System Gold), and the purified peptide was lyophilized.

**Structural predictions of D-2V NS1.** The amino acid sequence of the D-2V (strain PR159S1) NS1 protein was analyzed by using eight different computer algorithms (DPM, DSC, GOR4, HNNC, PHD, Predictor, SIMPA96, and SOPM), and a consensus structural prediction of the alpha-helix, 3_10-helix, Pi-helix, P-helix, P-helix, P-helix, and P-helix, extended-strand, β-turn, bend region, random-coil, or ambiguous states was assigned to each amino acid by using the Pole Bio-Informatique Lyonnais database (http://npsa-pbil.ibcp.fr). The amino acid sequences of the flavivirus NS1 proteins used in this study were obtained from the NCBI database (ncbi.nlm.nih.gov).
TABLE 1. Reactions of MAbs and PAbs generated to the D-2V NS1 with the NS1 proteins of the dengue viruses and other flaviviruses

| Antibodya | Specificityb (epitope) | ELISA titerc against the nonreduced (reduced) D-2V NS1 glycoprotein | Immunoblot reaction against nonreduced flavivirus NS1 glycoproteinsd |
|-----------|------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| MAb       |                        |                                                               |                                                               |
| 1H7.4     | D-2V NS1 (LD2) 6.1 (1.3) | +                                                             | ++                                                            |
| 1A12.3    | D-1V to D-4V NS1 (LX1) 3.0 (2.1) | +                                                             | + + + + + + + + + + + + + + + + + |
| 3D1.4     | D-1V to D-4V NS1 (LX1) 5.0 (4.1) | +                                                             | + + + + + + + + + + + + + + + + + |
| 4H3.4     | D-1V to D-4V NS1 (LX1) 4.3 (3.4) | +                                                             | + + + + + + + + + + + + + + + + + |
| 3A5.4     | D-1V to D-4V NS1 (LX1) 5.2 (4.7) | +                                                             | + + + + + + + + + + + + + + + + + |
| 1G5.4-A1-C3 | D-1V to D-4V NS1 (MLT) 5.1 (3.8) | +                                                             | + + + + + + + + + + + + + + + + + |
| PAbs      |                        |                                                               |                                                               |
| Rabbit    | D-1V to D-4V NS1 (MLT) 5.3 (4.4) | + + + + + + + + + + + + + + + + + |                                                               |
| Mouse     | D-1V to D-4V NS1 (MLT) 4.6 (2.8) | + + + + + + + + + + + + + + + + + |                                                               |
| Human     | D-1V to D-4V NS1 (MLT) 4.4 (3.3) | + + + + + + + + + + + + + + + + + |                                                               |

a MAbs generated to the D-2V NS1 protein or pools of PAbs generated to purified D-2V NS1 protein in outbred mice and rabbits and in human DSS patients during live D-2V infections.
b D-2V serotype-specific (D-2V NS1) or dengue virus complex reactive (D-1V to D-4V NS1) anti-dengue virus NS1 protein MAbs with the epitope name (in parentheses) or the reaction of a MAb or PAbs with multiple epitopes (MLT) on the dengue virus NS1 proteins.
c The reciprocal log_{10} titer against the nonreduced (reduced) D-2V NS1 protein.
d Immunoblot (Western blot) reactions against the nonreduced NS1 proteins of TBE virus, JE virus, WN virus, SLE virus, D-1V, D-2V, D-3V, D-4V, and YF virus, gauged by color intensities on an arbitrary scale ranging from negative (blank) to +++++.

NS1 proteins of each dengue virus serotype and other flaviviruses, as described previously (10). Some of these flaviviruses (e.g., JE, WN, and YF viruses) cocirculate in areas where dengue virus is endemic. For this study, representatives of flavivirus antigenic complexes I (TBE virus), III (JE, WN, and SLE viruses), VII (D-1V, D-2V, D-3V, and D-4V), and U (YF virus) were chosen. Since no common flavivirus group epitopes have been identified on the NS1 proteins, the replication of each virus was assessed by using MAb 4G2 and human PAbs which defined flavivirus group epitopes on the envelope proteins. By this method, all of these flaviviruses were found to have adequately replicated in the mammalian cells, and therefore, only minor adjustments in these flavivirus-infected cell lysate volumes were required to obtain similar strong immunoblot reactions. This method therefore ensured that high concentrations of each flavivirus NS1 protein were also present on these immunoblot strips (10). In this study, three of the MAbs (MAbs 1A12.3, 3D1.4, and 3A5.4) which defined epitope LX1 reacted with the D-2V NS1 protein in the ELISA in both the nonreduced and the reduced forms and displayed equal immunoblot reaction intensities against the nonreduced NS1 proteins of each dengue virus serotype (Table 1). MAb 4H3.4, which also defined the LX1 epitope, however, displayed an immunoblot reaction profile of D-1V = D-4V > D-2V = D-3V. MAbs 1A12.3, 4H3.4, and 3A5.4, but not MAb 3D1.4, also showed weaker cross-reactions with the NS1 proteins of one or more of the three antigenic complex III viruses tested (JE, WN, and SLE viruses); but none of these MAbs cross-reacted with the NS1 proteins of either the TBE or the YF virus, which are from other flavivirus antigenic complexes, as shown previously (10). In contrast, MAb 1G5.4-A1-C3, which defined multiple ELK/KLE-type motifs on the dengue virus NS1 proteins, displayed a D-2V > D-4V > D-1V > D-3V immunoblot reaction profile (6) and was nonreactive with the NS1 proteins of the other flaviviruses, as shown previously (6). The control MAb (MAb 1H7.4), which defined the LD2 epitope (11), reacted only with the NS1 protein of D-2V among this group of flaviviruses (Table 1). This MAb reaction was, however, abrogated by a reduction of this protein in the ELISA, unlike that observed in immunoblot assays (10, 11).

The PAbs to the purified D-2V NS1 protein generated in outbred rabbits displayed an immunoblot reaction profile of D-2V = D-4V > D-1V = D-3V and also displayed weak to moderate immunoblot cross-reactions with the NS1 proteins of the JE, WN, and SLE viruses from the antigenic complex III flaviviruses, as well as with that of YF virus. These patterns were therefore unlike any of the MAb reaction patterns with these viruses. The PAbs generated to the purified D-2V NS1 protein by mice and by human DSS patients, however, showed the same virus immunoblot reaction profile (D-2V > D-4V > D-1V > D-3V) to that of MAb 1G5.4-A1-C3. In addition, with the single exception of the weak cross-reaction of the human PAbs with the NS1 protein of JE virus, these mouse and human PAbs were also nonreactive with the NS1 proteins of the JE, WN, and SLE viruses, as observed with MAb 1G5.4-A1-C3. MAb 3D1.4, which defined the LX1 epitope, therefore strongly reacted with the NS1 proteins of each dengue virus serotype but did not react with any of the other flaviviruses. This MAb may therefore be useful for the specific diagnosis of dengue virus infection in areas where these other flaviviruses also cocirculate.

Reactions of mouse, human, and rabbit PAbs generated to the D-2V NS1 protein with linear (sequential) epitopes on this protein. To further compare the profiles of the PAb reaction to the purified D-2V NS1 protein generated in mice, rabbits, and human DSS patients, these PAbs were reacted with 174 overlapping 9-amino-acid peptides sequentially spanning the D-2V (strain PR159S1) NS1 protein sequence (Figure 1). In this study, both the mouse and the human PAbs strongly reacted with many epitopes previously identified by mouse MAbs (6, 7, 11) and showed antipeptide reaction profiles similar to those previously described by the use of MAb 1G5.4-A1-C3 (6). The following epitopes and peptides were all strongly identified by both the mouse and human PAbs: epitope LD2 (peptide 13,
The ELK/KLE-type epitopes appeared to be immunodominant in both the outbred mice and human DSS patients, but unlike those of the outbred mice, these human PAbs were generated to live D-2V infections. The titers of the PAbs to the ELK/KLE-type motifs in mice immunized with the purified D-2V NS1 protein were dependent upon major histocompatibility complex (H-2) class II molecules (6). The ability of these H-2 molecules to also affect the PAb responses to the D-2V NS1 protein during repeated infections with live D-2V in mice was therefore tested. In this study, three mice of each congenic strain (strain B10.G, I-A<sup>d</sup>; strain B10.RIII, I-A<sup>d</sup>, I-E<sup>d</sup>; strain B10.M, I-A<sup>f</sup>; strain B10.S, I-A<sup>s</sup>; strain C57BL/BJ, I-A<sup>b</sup>; strain B10.BR, I-A<sup>b</sup>, I-E<sup>b</sup>; strain B10.A, I-A<sup>b</sup>; and strain B10.D2N, I-A<sup>d</sup>, I-E<sup>b</sup>) were infected twice with live D-2V and the sera were collected 2 weeks after the first and second infections (Figure 2). When a 50% endpoint ELISA cutoff titer (log<sub>10</sub> <i>t</i><sub>50</sub>) of 1.0 was applied to each serum sample obtained after the first D-2V infection, only the strain B10.RIII mice were identified to be high responders (for all mice, log<sub>10</sub> <i>t</i><sub>50</sub> > 1.0), while the strain B10.BR and B10.A mice were identified to be low responders (for all mice, log<sub>10</sub> <i>t</i><sub>50</sub> < 1.0). When a log<sub>10</sub> <i>t</i><sub>50</sub> of 2.0 was applied to each serum sample collected after the second D-2V infection, the B10.RIII (I-A<sup>d</sup>, I-E<sup>d</sup>), B10.S (I-A<sup>s</sup>), and C57BL/BJ (I-A<sup>b</sup>) mouse strains were identified to be high responders (for all mice, log<sub>10</sub> <i>t</i><sub>50</sub> > 2.0), while the B10.BR (I-A<sup>b</sup>, I-E<sup>b</sup>) and B10.A (I-A<sup>b</sup>) mice were again identified to be low responders (for all mice, log<sub>10</sub> <i>t</i><sub>50</sub> < 2.0). The antibody responses generated to the D-2V NS1 protein during live D-2V infections were therefore influenced by H-2 class II molecules, and the same high- and low-responder class II haplotypes were identified in response to live D-2V infections as to immunizations with the purified D-2V NS1 protein (6).

Cross-reaction of PAbs generated to live D-2V in congenic mice with human platelets and fibrinogen. Since MAbs 1G5.4-A1-C3 and PAbs generated in mice immunized with the purified D-2V NS1 protein cross-reacted with human fibrinogen and integrin/adhesion molecules on human platelets (6), the PAb pools generated in mouse strains that were high (strains B10.RIII, B10.S, and C57BL/BJ), moderate (strains B10.D2N), and low (strains B10.BR and B10.A) responders to the live D-2V infections according to their ELISA titers were also tested against these human antigens (Figure 3). Even though...
they displayed different ELISA titers against the D-2V NS1 protein, the PAbs from all of these mouse strains had similar ELISA titers against human platelets and fibrinogen, while they only weakly cross-reacted with human serum and chicken egg albumins. The strains that were moderate (strain B10.D2N) and low (strains B10.BR and B10.A) responders therefore generated higher (hetero-specific) ELISA titers against human platelets and fibrinogen than against the D-2V NS1 protein, while antibodies from the control B10.S mice [B10.S(C)] immunized with virus-free growth medium, poorly cross-reacted with these human proteins. Live D-2V infections in mice therefore generated strongly cross-reactive PAbs against epitopes on human fibrinogen and platelets, as was observed after immunizations of mice with the purified D-2V NS1 protein (6) and MAb 1G5.4-A1-C3 (6).

Precise mapping of dengue virus complex epitope LX1 on D-2V NS1 protein using mouse MAbs. Because the four MAbs which defined the LX1 epitope showed different reaction patterns with the NS1 proteins of different flaviviruses (Table 1), precise mapping was performed to identify the minimum core LX1 amino acid sequence that was specifically defined by each of these MAbs. MAbs 1A12.3, 3D1.4, 4H3.4, and 3A5.4, which reacted with peptides 56 and 57 among the 174 overlapping synthetic peptides spanning the D-2V NS1 protein sequence, as reported previously (11), were further tested by using a set of sequential peptides spanning this immunodominant region (amino acids 105 to 129) of the protein and a set of sequentially truncated LX1 peptides (Table 2). In this study, MAbs 4H3.4 and 3A5.4 reacted the most strongly with peptide 56 (111LRYSWKTWG119), while MAbs 1A12.3 and 3D1.4 reacted the most strongly with peptide 57 (113YSWKTWGKA121). In contrast, MAb 1G5.4-A1-C3 reacted with peptides 53, 54, and 55, which were also strongly identified by the outbred mice and human DSS patients (Figure 1), each of which contained the 110ELR112 sequence (ELK-type motif) (Table 2). Of these three peptides, peptide 55 (110TELRYSWKT117) was the most strongly identified by this MAb.

When MAbs 3D1.4, 4H3.4, and 3A5.4 were reacted with a set of sequentially truncated peptides within the 113- to 121-amino-acid sequence, these MAbs showed stronger reactions against the core 7-amino-acid sequence (113YSWKTWG119) than either of the longer peptides (peptides 56 and 57), while MAbs 1A12.3 and 1G5.4-A1-C3 reacted poorly with these truncated peptides. MAb 1A12.3 therefore required the full 9-amino-acid sequence of peptide 57 (113YSWKTWGKA121) for optimal binding. The core 7-amino-acid LX1 sequence (113YSWKTWG119) was conserved in the NS1 proteins of both D-3V and D-4V, but the corresponding NS1 protein sequence...
of D-IV (113YSWKS\textsubscript{GW}119) contained a replacement of 116T by S that was shown to be antigenically silent (11) (Figure 4). The corresponding NS1 protein sequences from the TBE (YSWK\textsubscript{SW}GW), JE and WN (MGWK\textsubscript{KW}GW), and YF (YGKW\textsubscript{T}KWG) viruses contained other amino acid substitutions (underlined). The lack of cross-reactivity of these mouse MAbs with the NS1 protein of TBE virus was therefore possibly due to the replacement of 116Y by V. The corresponding NS1 sequences of JE and WN viruses were, however, identical. These core 7-amino-acid sequences per se could not therefore account for the weak reaction of MAbs 1A12.3, 3D1.4, 4H3.4, and 3A5.4 (or multiple ELK/KLE-type motifs (MAbs 1G5.4-A1-C3)) with the NS1 protein and a set of peptides in which the LX1 epitope (113YSWKTGWG\textsubscript{KA}) was sequentially truncated. \(A_{opt}\) values of >100 for each MAb are underlined, and the peak reactions against each overlapping and truncated synthetic peptide set are shown in boldface.

**TABLE 2.** Precise mapping of the LX1 epitope of the dengue-2 virus NS1 protein with MAbs.

| Peptide sequence | ELISA reaction (\(A_{opt}\)) to MAbs: |
|------------------|-------------------------------------|
|                   | 1A1A2.3 | 3D1.4 | 4H3.4 | 3A5.4 | 1G5.4-A1-C3 |
| RQPTELRLY        | 0.24    | 0.07  | 0.05  | 0.04  | 1.15        |
| QPTELRLYSN       | 0.50    | 0.08  | 0.17  | 0.05  | 1.04        |
| TELRYSKWT       | 0.42    | 0.06  | 0.12  | 0.13  | 1.24        |
| LRYYSKWTGW       | 0.19    | 1.20  | 1.37  | 1.70  | 0.74        |
| YYSKWTGWKA       | 1.73    | 1.75  | 1.22  | 1.29  | 0.42        |
| WKTGWKAM        | 0.79    | 0.87  | 0.78  | 0.20  | 0.16        |
| TWCATCHAELS      | 0.03    | 0.09  | 0.05  | 0.01  | 0.05        |
| GKKAKMELSTE      | 0.02    | 0.06  | 0.04  | 0.01  | 0.04        |
| AKMLSTELHL      | 0.09    | 0.07  | 0.04  | 0.03  | 0.25        |
| YYSKWTGW         | 0.14    | 1.09  | 0.70  | 0.80  | 0.19        |
| YYSKWTW          | 0.21    | 2.49  | 2.10  | 1.83  | 0.16        |
| YYSKWTW          | 0.15    | 1.92  | 0.54  | 1.22  | 0.18        |
| YYSKWT          | 0.09    | 0.12  | 0.09  | 0.08  | 0.14        |
| SWKTGWKA        | 0.47    | 1.19  | 0.98  | 1.05  | 0.20        |
| WKTGWKA         | 0.87    | 0.97  | 0.36  | 0.58  | 0.18        |
| KTKGW          | 0.46    | 0.08  | 0.09  | 0.08  | 0.17        |
| TNGKA          | 0.11    | 0.12  | 0.10  | 0.09  | 0.09        |

*MAbs, which defined either the LX1 epitope (MAbs 1A12.3, 3D1.4, 4H3.4, and 3A5.4) or multiple ELK/KLE-type motifs (MAbs 1G5.4-A1-C3), were diluted 1/250 to 1/500 and reacted with overlapping synthetic peptides sequentially spanning amino acids 105 to 129 of the D-2V (strains PR159S1 and TR1751) NS1 protein, and a set of peptides in which the LX1 epitope (113YSWKTGWK\textsubscript{A}) was sequentially truncated. \(A_{opt}\) values of >100 for each MAb are underlined, and the peak reactions against each overlapping and truncated synthetic peptide set are shown in boldface.

**Design of AFLX1 peptide.** The abilities of the LX1 and ELK-type epitopes in this immunodominant region of the protein to be faithfully represented within a synthetic peptide when it was bound in solid-phase immunoassays and to also generate antibody responses in mice when the appropriate H-2 class II (T-helper) epitope was included were tested. For this study, epitope LX1 containing the flanking 6-amino-acid sequence 121AKMLST\textsubscript{126} was prepared. The 121AKMLST\textsubscript{126} sequence was predicted to strongly bind to the H-2 (class II) I-A\(^d\) molecule because of its maximum cumulative amino acid score of 729 (27) (Figure 4). This AFlX1 peptide was capped by a natural glutamic acid (\(1^{\text{110}}\)E) residue at the amino terminus to include the ELR sequence (ELK-type motif) with a natural histidine (\(1^{\text{120}}\)H) and an unnatural cysteine residue at the carboxyl terminus to allow cysteine-bridged dimer formation. By using the consensus results from eight computer algorithms, the LX1 epitope region (111LRYSKWTGWG\textsubscript{119}) was predicted to contain amino acid residues in extended-strand and random-coil arrangements, while the putative I-A\(^d\) binding sequence was predicted to be in an alpha-helical conformation. An I-A\(^d\) binding motif was also predicted in the corresponding sequence of the D-3V NS1 protein (121AKIVTA\textsubscript{126}) because of its maximal cumulative score of 729, but such a motif was not predicted for the corresponding sequences of the D-1V and D-4V NS1 proteins (cumulative scores, <400).

**Immunoblot (Western blot) assay and ELISA with the AFlX1 peptide.** The ability of the AFlX1 peptide to optimally display the LX1 and ELK-type epitopes was tested in an ELISA and an immunoblot assay. The predicted molecular mass of the AFlX1 peptide was 2.506 kDa, but it could also exist as a cysteine-bridged homodimer. Immunoblot assays showed that this peptide was present in both the monomeric and dimeric forms, and it could more efficiently be detected on nylon membranes rather than on nitrocellulose membranes when it was nonreduced (Figure 5). At optimal nonreduced AFlX1 peptide concentrations of 8 \(\mu\)g for the immunoblot assay and an 8-\(\mu\)g/ml coating concentration determined for the ELISA, the four MAbs which defined epitope LX1 and MAbs 1G5.4-A1-C3, which defined ELK-type motifs, all strongly reacted with this peptide in both of these assays (Table 3). MAb 1A12.3, which required the entire 9-amino-acid sequence of peptide 57 for optimal binding (Table 2), uniquely had a slightly higher ELISA titer (4.0 times) against the AFlX1 peptide (log\(_{10}\) t\(_{50}\) 3.6) than the native, dimeric D-2V NS1 peptide.

![FIG. 4.](https://example.com/fig4) Design of AFlX1 peptide. The AFlX1 peptide contained the 110- to 129-amino-acid sequence of the D-2V (strain PR159S1 and TR1751) NS1 protein, with the LX1 epitope (shown in boldface), the 121AKMLST\textsubscript{126} sequence predicted to be an H-2 I-A\(^d\) binding motif (cumulative score, >400) (27), a natural glutamic acid (E) (negative [\(-ve\)] charge) residue at the amino terminus and natural histidine (H) (positive [\(+ve\)] charge), and an unnatural cysteine (C) residue at the carboxy terminus. The molecular shape (C, random coil; H, alpha helix; E, extended strand; X, no consensus) predicted by using the consensus results from eight computer algorithms, is shown. The amino acid substitutions which occur within the LX1 epitope from the NS1 proteins of D-1V, D-3V, and D-4V are underlined, and the cumulative I-A\(^d\) binding scores in their corresponding 6-amino-acid sequences are shown.
protein (log_{10} t_{50}, 3.0) (Table 3). MAb 1G5.4-A1-C3 showed similar titers against both the AFLX1 peptide (log_{10} t_{50}, 4.9) and the native NS1 protein (log_{10} t_{50}, 5.1), while the other three MAbs, which defined the core 7-amino-acid LX1 epitope sequence (Table 2), all had slightly lower ELISA titers (3.2 times) against the AFLX1 peptide than the D-2V NS1 protein (Table 3). The control, MAb 1H7.4, which defined epitope LD2, was, as expected, nonreactive with the AFLX1 peptide in the ELISA and the immunoblot assay. Both the LX1 and the ELK-type epitopes were therefore suitably displayed in the AFLX1 peptide when it was bound in both of these solid-phase immunoassays.

**Immunogenicity and antigenicity of the AFLX1 peptide and the epitopes within its sequence.** Since both the LX1 and ELK-type epitopes were adequately exposed in the AFLX1 peptide, their relative immunodominances were compared in congeneric mouse strains. The immunogenicity of this peptide was tested in I-A\(^d\)-positive mice, for which the AFLX1 peptide contained a binding motif, and I-A\(^d\)-positive (control) mice. The PAb responses of these mice were then compared by using the same congeneric mouse strains immunized with the purified D-2V NS1 protein or with live D-2V by using the overlapping and truncated sets of peptides used to map the LX1 epitope (Table 2), the AFLX1 peptide, and the purified D-2V NS1 protein. The number of recycles of these peptide-coated pins/gears was, however, limited, and individual serum samples from each group of congeneric mice showed only minor variations in either their ELISA titers against the purified D-2V NS1 protein or their immunoblot reaction profiles against the NS1 proteins of each dengue virus serotype (6). These reactions were therefore performed by using pools of PAbs from these congeneric mice before these Pab and Mab (Table 2) reactions were compared with those of individual serum samples from panels of DF and DSS patients. In this study, strain B10.D2N (I-A\(^d\), I-E\(^d\)) and strain B10.S (I-A\(^d\)) mice were immunized with 50 \(\mu g\) of the AFLX1 emulsified in adjuvant and boosted with the same dose. Pools of PAbs from these B10.D2N mice developed a moderately high antibody ELISA titer (log_{10} t_{50}, 2.9) against the AFLX1 peptide, but they were only weakly reactive against the native, dimeric D-2V NS1 protein (log_{10} t_{50}, 1.7) (Table 4). Although they were of lower absorbance intensities, the antibody reaction profiles of these B10.D2N mice against the set of overlapping peptides covering the AFLX1 peptide sequence were similar to those generated in the mouse strains that were medium (strain B10.D2N) and high (strain B10.S) responders either to the purified D-2V NS1 protein or to live D-2V infections. The strongest antibody reaction of these mouse PAbs was against peptides 53, 54, and 55, which contained the \(110^{\text{ELR}}112\) (ELK-type motif), with the peak reaction being against peptide 55 (109\(\text{TELRYSWKT}^{117}\)), as observed with MAb 1G5.4-A1-C3 (Table 2). No reaction against the core LX1 epitope sequence (113\(\text{YSWKTG}^{119}\)) was, however, observed by using these B10.D2N mouse PAbs (Table 4). Thus, even though these antibodies generated a high ELISA titer (log_{10} t_{50}, 2.9) against the AFLX1 peptide in these mice, the antibodies were predominantly not directed against these sequential epitopes within this peptide. In contrast, AFLX1 was nonimmunogenic in the B10.S mice, as well as C57BL/BJ (H-2 class II, I-A\(^b\)) mice (data not shown), and, therefore, the I-A\(^d\)-binding motif identified in the AFLX1 peptide sequence accounted for its immunogenicity in these B10.D2N (H-2 class II, I-A\(^d\)) mice.

B10D2N and B10.S mice immunized with the purified D-2V NS1 protein generated high ELISA titers against the D-2V NS1 protein (log_{10} t_{50}s, 4.0 and 4.7, respectively), and their peak reactions against peptide 55 (109\(\text{TELRYSWKT}^{117}\)), which contained the ELK-type motif (underlined) and the core LX1 peptide (113\(\text{YSWKTG}^{119}\)), were reflected in their ELISA titers (log_{10} t_{50}s, 2.4 and 2.9, respectively) and immunoblot reactions (color intensities, + and ++, respectively) against the AFLX1 peptide. Because these B10.D2N mice generated only a weak reaction against the core LX1 epitope (absorbance, 0.43), their antibody reactions against the AFLX1 peptide in the immunobassays were therefore predominantly due to their reaction with the immunodominant ELR sequence (ELK-type motif).

Pools of PAbs from B10D2N and B10.S mice repeatedly infected with live D-2V showed the same peak reactions against peptide 55 and the core LX1 epitope, but the reactions

**TABLE 3. Comparative ELISA and immunoblot reactions of mouse MAbs against the purified D-2V NS1 protein and the AFLX1 peptide**

| MAb   | Epitope | ELISA titer (log_{10} t_{50}) | Immunoblot reaction |
|-------|---------|-------------------------------|---------------------|
|       | NS1 protein | AFLX1 peptide | NS1 protein | AFLX1 peptide |
| 1A12.3 | LX1      | 3.0  | 3.6 | ++  | ++  |
| 3D1.4  | LX1      | 5.0  | 4.5 | ++  | ++  |
| 4H3.4  | LX1      | 4.3  | 3.4 | ++  | ++  |
| 3A5.4  | LX1      | 5.2  | 4.3 | ++  | ++  |
| 1G5.4-A1-C3 | MULTIPLE | 5.1  | 4.9 | ++  | ++  |
| H7.4 (control) | LD2 | 6.1  | <1.5 | +++ | +++ |

\(a\) Epitope name (LD2 or LX1) or reaction with multiple (MULTIPLE) epitopes on the D-2V NS1 protein.

\(b\) Reciprocal log_{10} t_{50} against the purified D-2V NS1 protein and the AFLX1 peptide.

\(c\) Immunoblot color reaction intensities of the MAbs against the nonreduced forms of the purified D-2V NS1 protein and the AFLX1 peptide, gauged on an arbitrary scale ranging from negative (blank) to +++. 

**FIG. 5. Immunoblot of the nonreduced and reduced AFLX1 peptide on nitrocellulose and nylon membranes.** Two concentrations (10 and 25 \(\mu g\)) of the AFLX1 peptide were nonreduced (lanes −) or reduced (lanes +) with 2-mercaptoethanol (2ME), subjected to 15% (wt/vol) SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose or nylon membranes, and detected with MAb 3D1.4. The locations of the monomer (2.5-kDa) and dimer (5.0-kDa) forms of the peptide are shown.
were lower than those generated by the same congenic mouse strains immunized with the purified D-2V NS1 protein. Thus, these peak antibody reactions of the B10.D2N mice with peptide 55 and the core LX1 epitope sequence were both below an absorbance of 1.0, while only the reaction of the B10.S mouse sera with peptide 55 was above this value. These results were reflected in their lower ELISA titers against the purified D-2V NS1 protein and the AFLX1 peptide sequence in both the ELISA (log10 A50 of 1.06) and the immunoblot assay at 1/50 dilutions. The results with the overlapping and truncated peptides are expressed as A50 values of >1.00 are underlined, and the peak reactions with each of these peptide sets are shown in boldface. The ELISA results are expressed as the reciprocal log10 A50 and the immunoblot color reaction intensities are gauged on an arbitrary scale ranging from negative (blank) to ++ + for comparison with the results in Table 5.

The ELK-type motifs present in peptides 53, 54, and 55 were therefore immunodominant in all of these mice. These mouse PABs and MAB 1G5.4-A1-C3 therefore defined the same ELK/KLE-type epitopes in mice immunized with either the AFLX1 peptide, the purified D-2V NS1 protein, or live D-2V were reacted with the sets of synthetic peptides at 1/75 to 1/125 dilutions, the purified D-2V NS1 protein in an ELISA, and the AFLX1 peptide in an ELISA and in an immunoblot assay at 1/50 dilutions. The results with the overlapping and truncated peptides are expressed as A50 values of >1.00 are underlined, and the peak reactions with each of these peptide sets are shown in boldface. The ELISA results are expressed as the reciprocal log10 A50 and the immunoblot color reaction intensities are gauged on an arbitrary scale ranging from negative (blank) to ++ + for comparison with the results in Table 5.

Antigenicity of the AFLX1 peptide and the epitopes within its sequence using human PABs. To further support the role of anti-ELK/KLE-type antibodies in the pathogenesis of DHF/DSS, quantitative and qualitative differences against these epitopes were tested by using PAB samples from patients with mild (DF) and severe (DSS) disease. In this study, individual serum samples from panels of patients with DF (n = 3) and DSS (n = 3) showed similar reaction profiles, with the strongest reactions being against peptides 53, 54, and 55 and with the peak reaction being against peptide 55 (Table 5), as shown by MAB 1G5.4-A1-C3 (Table 2) and the mouse PABs generated to either the purified D-2V NS1 protein or live D-2V (Table 4). These antibody reactions were, however, stronger in each of the patients with DSS (absorbances, 1.43, 1.49, and 1.72) than DF (absorbances, 0.82, 0.87, and 0.91) (Table 5). In addition, peptides 56 and 57, which contained the LX1 epitope as well as the core LX1 epitope sequence (113YSWKTWG119), were much more strongly identified by the PABs from each of the DSS patients than by those from the DF patients. These differences were also reflected in their reactions against the purified D-2V NS1 protein in the ELISA (for the DF patients, log10 A50s of 2.4, 2.6, and 2.8) and against the AFLX1 peptide in both the ELISA (for the DF patients, log10 A50s of 1.6, 1.7, and 1.9; for the DSS patients, log10 A50s of 2.4, 2.6, and 2.8) and the immunoblot assay (all DF patients were immunoblot negative; for the DSS patients, color intensities of + + + for comparison with the results in Table 5).

In conclusion, both mice and humans generated immunodominant antibody responses to the ELK/KLE-type motifs in the D-2V NS1 protein during live D-2V infections, but these were much stronger in each of the patients with DSS than in those with DF.

TABLE 4. Antibody reactions generated in congenic mice to either the AFLX1 peptide, the purified D-2V NS1 protein, or repeated infections with D-2V against overlapping and truncated sets of synthetic peptides within the AFLX1 sequence, the AFLX1 peptide, and the purified D-2V NS1 protein

| AFLX1 peptide sequence | A50 for sera from mouse strains immunized with the following immunogen |
|-------------------------|---------------------------------------------------------------|
|                         | AFLX1 peptide | D-2V NS1 | Live D-2V |
|                         | B10.D2N | B10.S | B10.D2N | B10.S | B10.D2N | B10.S |
| 110ELRYSWKTWGKKAMLSTELHC | 0.57 | 0.37 | 0.96 | 1.35 | 0.70 | 1.03 |
| RPQTTELEY              | 0.43 | 0.34 | 0.91 | 1.31 | 0.65 | 0.94 |
| QPTELEYSN              | 0.68 | 0.34 | 1.19 | 1.41 | 0.79 | 1.14 |
| TELRYSWKTG             | 0.43 | 0.23 | 0.42 | 1.06 | 0.36 | 0.48 |
| YSWKTVG                 | 0.27 | 0.20 | 0.40 | 0.89 | 0.30 | 0.41 |
| YSWKTYGKA              | 0.19 | 0.17 | 0.31 | 0.66 | 0.25 | 0.35 |
| KTWKGKAM                | 0.18 | 0.14 | 0.28 | 0.53 | 0.21 | 0.25 |
| TWGKAMLST               | 0.17 | 0.12 | 0.22 | 0.35 | 0.17 | 0.23 |
| AMMLSTELH              | 0.37 | 0.16 | 0.56 | 0.88 | 0.37 | 0.45 |
| YSWKTVGK               | 0.20 | 0.17 | 0.31 | 0.72 | 0.28 | 0.34 |
| YSWKTVW                | 0.18 | 0.16 | 0.43 | 1.26 | 0.35 | 0.68 |
| YSWKTVT               | 0.21 | 0.15 | 0.24 | 0.73 | 0.22 | 0.43 |
| YSWKTV               | 0.17 | 0.14 | 0.20 | 0.64 | 0.18 | 0.22 |
| WTVKXGKA              | 0.2 | 0.17 | 0.24 | 0.47 | 0.21 | 0.25 |
| WTVKXGKA              | 0.16 | 0.14 | 0.18 | 0.42 | 0.19 | 0.31 |
| KTVSKGKA             | 0.21 | 0.11 | 0.52 | 0.53 | 0.39 | 0.37 |
| TVKSKGKA            | 0.38 | 0.13 | 0.46 | 0.88 | 0.21 | 0.66 |
| Anti-D-2V NS1 ELISA titer (log10 A50) | 1.7 | 1.2 | 4.0 | 4.7 | 1.7 | 2.3 |
| Anti-AFLX1 peptide ELISA titer (log10 A50) | 2.9 | 1.3 | 2.4 | 2.9 | 1.6 | 2.1 |
| Immunoblot against the AFLX1 peptide | + | + + | + |
TABLE 5. Antibody reactions generated in human patients with DF and DSS against sets of overlapping and truncated synthetic peptides within the AFLX1 sequence, the AFLX1 peptide, and the purified D-2V NS1 protein

| AFLX1 peptide sequence<sup>a</sup> | DF | DSS |
|----------------------------------|----|-----|
|                                  | Patient 1 | Patient 2 | Patient 3 | Patient 1 | Patient 2 | Patient 3 |
| RPQTTELRY                        | 0.81 | 0.80 | 0.83 | 1.13 | 1.25 | 1.45 |
| QPTTELRYW                       | 0.74 | 0.74 | 0.76 | 1.11 | 1.21 | 1.48 |
| TELEXYSKT                        | 0.82 | 0.87 | 0.91 | 1.43 | 1.49 | 1.72 |
| LRYSWXWG                        | 0.42 | 0.48 | 0.62 | 0.86 | 1.16 | 1.33 |
| YSWKTXWGK                       | 0.37 | 0.43 | 0.50 | 1.01 | 1.06 | 1.27 |
| WKTGXKMK                        | 0.32 | 0.38 | 0.42 | 0.78 | 0.94 | 1.02 |
| TWGXKML                          | 0.20 | 0.30 | 0.34 | 0.73 | 0.87 | 0.95 |
| GAKMLSTE                         | 0.21 | 0.24 | 0.35 | 0.60 | 0.64 | 0.83 |
| AKMLSTELH                        | 0.43 | 0.56 | 0.51 | 0.77 | 0.96 | 1.27 |
| YSWKTXWGK                       | 0.32 | 0.37 | 0.43 | 0.83 | 0.98 | 1.12 |
| YSWKTXWG                         | 0.28 | 0.34 | 0.48 | 1.01 | 1.03 | 1.03 |
| YSWKTXW                          | 0.20 | 0.25 | 0.31 | 0.69 | 0.89 | 0.95 |
| YSWKTX                           | 0.20 | 0.23 | 0.27 | 0.41 | 0.55 | 0.81 |
| SWKTXWGK                        | 0.26 | 0.31 | 0.34 | 0.72 | 0.57 | 0.95 |
| WKTXWGK                         | 0.22 | 0.25 | 0.32 | 0.42 | 0.45 | 0.97 |
| KTWXGK                          | 0.20 | 0.22 | 0.35 | 0.66 | 0.48 | 0.88 |
| TWGXK                           | 0.27 | 0.31 | 0.54 | 0.87 | 0.64 | 1.05 |

<sup>a</sup> Peptide sequences of a set of overlapping 9-amino-acid synthetic peptides sequentially spanning the AFLX1 peptide sequence and a set of sequentially truncated (5- to 9-amino-acid) peptides. The ELK-type motifs are underlined, and the two 9-amino-acid peptides containing the LX1 epitope and the core 7-amino-acid LX1 epitope are shown in boldface.

<sup>b</sup> Individual serum samples, obtained 4 to 6 days after the onset of symptoms from patients with either DF (n = 3) or DSS (n = 3), were reacted with the sets of synthetic peptides at 1/75 to 1/125 dilutions, the purified D-2V NS1 protein in an ELISA, and the AFLX1 peptide in an ELISA and an immunoblot assay at 1/50 dilutions. The results with the overlapping and truncated peptides are expressed as A<sub>492</sub> values of > 1.00 are underlined, and the peak reactions with each of these peptide sets are shown in boldface. The ELISA results are expressed as the reciprocal log<sub>10</sub> titers, and the immunoblot color reaction intensities are gauged on an arbitrary scale ranging from negative (blank) to +++.

DISCUSSION

The immunodominance of the ELK/KLE-type epitopes accounted for the similar antibody reaction profiles of MAb 1G5.4-A1-C3 and the PAbs generated in mice and humans against peptides spanning the immunodominant region of the D-2V NS1 protein and the D-2V > D-4V > D-1V > D-3V reaction patterns observed against the NS1 proteins of each dengue virus serotype (6, 10). It was previously shown that rabbits, unlike humans and mice, generated antibodies to the carboxy-terminal region of a recombinant D-2V NS1 protein (25). While the rabbit sera used in this study reacted only weakly with some of the short peptide sequences in the carboxy-terminal region of the D-2V NS1 protein sequence, these epitopes may be partially or totally dependent upon the protein conformation. The results from the other study (25) may have, however, accounted for the greater sensitivity of rabbit anti-dengue virus NS1 protein sera in a dengue virus NS1 capture ELISA with mouse MAbs with defined epitopes (e.g., MAbs LD2, 2A4, and LX1) at the amino-terminal region of the protein (30) than in the assay with mouse PAbs (1), which would also have reacted with these immunodominant aminoterminal epitopes on the protein. In the latter study (1), increased sensitivity was achieved by using mouse PAbs for both the capture and the detection of the dengue virus NS1 protein. The results from this study, however, suggest that such an assay with either mouse or human PAbs could be used only with patient serum samples, from which the blood-clotting factors, such as fibrinogen, were completely removed. All of the problems of these dengue virus NS1 protein capture assays were, however, readily circumvented by using a simple and sensitive dot blot assay with MAb 3D1.4, coupled with a method to disrupt the patients’ immune complexes containing the dengue virus NS1 protein (18). MAb 3D1.4 was unique among the four MAbs which defined the LX1 epitope, since it strongly reacted with the NS1 proteins of each dengue virus serotype, which cocirculate in many countries in the world, but not with the NS1 proteins of any of the three antigenic complex III flaviviruses (e.g., JE, WN, and SLE viruses) tested in this study or with the Murray Valley encephalitis, Usutu, and Kunjin viruses, which are also from this antigenic complex (data not shown). MAb 3D1.4 will therefore be very useful for the specific detection of dengue viruses in areas where these other flaviviruses also cocirculate, preferably by the use of such a simple dot blot assay (18).

Although the dengue virus NS1 protein was not found to be an immunodominant protein during dengue virus infections of humans compared with the immunodominance of other viral proteins (e.g., the main envelope protein [E] and NS3 proteins) in immunoblot assays (3), the antibody responses to the dengue virus NS1 protein were detected during primary dengue virus infections in humans by using isotype-capture ELISAs (28). Similar results were obtained with mice in this
study, since the IgG antibody responses generated to the D-2V NS1 protein, particularly in the congeneric mouse strains that were high responders (e.g., strain B10.RIII), could be detected 2 weeks after the primary D-2V infection. Most of these congeneric mouse strains, however, generated much lower titers of antibodies to the D-2V NS1 protein after live D-2V infections than with the purified D-2V NS1 protein administered in adjuvant (6). Strain B10.RIII (I-A^k, I-E^k) mice, which possessed both the I-A and the I-E molecules, however, generated a mean antibody ELISA titer to the D-2V NS1 protein after two live D-2V infections that was only eightfold lower (mean log_{10} t_{50}, 2.8) than that after immunizations with the purified D-2V NS1 protein (mean log_{10} t_{50}, 3.7) (6). These mice therefore generated particularly high antibody responses to this protein after live D-2V infections, as was found by the use of the DSS patients’ PAbs. Further studies are needed to identify whether these results were due to either the I-A’ or the I-E’ molecule or to both of these H-2 class II molecules. In contrast, the strain B10.BR (I-A^k, I-E^k) mice, which also possessed both I-A and I-E molecules, generated lower antibody responses to the D-2V NS1 protein after live D-2V infections than the strain B10.A (I-A^k) mice, which expressed only the I-A molecule. While either the I-E or the I-A molecule has been shown to suppress the generation of autoantibodies to different antigens (4, 16), these B10.BR mice still generated highly cross-reactive antibody ELISA titers to the human platelets and fibrinogen after live D-2V infections (Figure 3) or immunizations with the purified D-2V NS1 protein (6). The HLA-DR4 molecule, positively selected in the Latin American populations, was associated with DHF resistance (20), while the HLA-DQ1 molecule provided DHF resistance in Brazilian populations (24). During a large DHF epidemic in Cuba, some diseases known to be associated with particular HLA class II haplotypes, such as bronchial asthma, were identified as DHF risk factors (15). Further studies are therefore needed to confirm these likely HLA class II haplotype associations with DHF/DSS, for which the AFLX1 peptide, which adequately displayed both the ELK-type and LX1 epitopes, may be a useful inexpensive diagnostic antigen.

The reaction of the human and mouse PAbs with the three peptides (amino acids 1 to 9, 3 to 11 and 5 to 13) at the amino terminus of the D-2V NS1 protein was consistent with the report that the amino acid sequence \( ^{1}DSGCVSVKNKEL \) is immunodominant in humans (17). In this study, stronger human and mouse antibody reactions were, however, observed against the three overlapping peptides (peptides 53, 54, and 55) which contained the immunodominant ELR (ELK-type motif). MAb 1G5.4-A1-C3, which defined the ELK/KLE-type motifs, identified similar antipeptide reaction peaks as those of human DSS patients and mice repeatedly infected with D-2V. This MAb produced intraperitoneal hemorrhage in mice and cross-reacted with human fibrinogen, platelets, and endothelial cells (6), while the PAbs to live D-2V infections generated in the mice also cross-reacted with human fibrinogen and platelets. The similar results obtained between the mouse and the human PAbs and MAb 1G5.4-A1-C3 are therefore likely to account for the ability of DHF/DSS patients’ PAbs to cross-react with human platelets (26) and endothelial cells (21) and the identification of IgG, IgM, and complement proteins with fibrinogen as a major autoantigen in DSS patients’ high-molecular-weight circulating immune complexes (2, 7). This would further account for the correlation of clinically graded dengue viral disease severity with the levels of the vasoactive compounds C3a and histamine (2) and the antibody-stimulating cytokine IL-6 (12, 13) and the inverse correlation with the plasma fibrinogen concentrations (2).

While antibodies generated to the purified D-2V NS1 protein cross-reacted with a human endothelial cell line in vitro, their reaction was reduced by a low concentration (50 \( \mu \)g/ml) of human fibrinogen (6). Thus, at the normal blood concentrations of human fibrinogen (1,600 to 4,200 \( \mu \)g/ml) and other blood proteins also containing ELK-type epitopes (6), these cross-reactions with epitopes on human endothelial cell integrin/adhesion molecules (e.g., \( \alpha V \beta 3 \) and ICAM-1) (6) would be dramatically reduced or possibly abrogated. In a subsequent study, PAbs generated to the dengue virus NS1 protein were shown to cross-react with human endothelial cells and to cause apoptosis (21). These reactions were, however, also performed in vitro with human cell lines under nonphysiological conditions. Similarly, while dengue virus infected human endothelial cells and caused damage in vitro, there is no evidence of this type of vascular damage in DHF/DSS patients (2). An animal model is therefore urgently required to more adequately confirm the roles of these autoantibodies in vascular leakage. A model for dengue virus antibody-enhanced disease in vivo was developed in mice. In that model, greater than 100,000 times antibody-enhanced replication of dengue virus was demonstrated (7), and in that model, these immunodominant anti-ELK/KLE-type epitope autoantibodies and other components of inappropriate immune activation implicated in DHF/DSS pathogenesis (2, 12, 13, 15) can be more relevantly studied. The results from these studies therefore suggest that the dengue virus NS1 proteins would be unsuitable for use as a vaccine against the dengue viruses, as originally proposed, and that a suitable live attenuated vaccine or subunit vaccine containing any of the other dengue virus proteins must never generate either autoantibodies to these immunodominant ELK/KLE-type motifs or an antibody-enhanced replication of these viruses (7). Further work is required to assess whether MAb 1G5.4-A1-C3 can generate enhanced disease by enhancing the replication of these viruses in vivo and also differentiate between virulent and less virulent strains of each dengue virus serotype.

Since hetero-specific antibodies to human autoantigens may be generated during dengue virus infections, as shown in this study, antibody reactions to the dengue virus NS1 proteins or the AFLX1 peptide per se may be unsuitable for use for the identification of dengue virus-infected patients during the early acute phase of disease who subsequently develop DHF/DSS. Such an assay was described previously (7) and is being further tested.

ACKNOWLEDGMENTS

This work received financial support from the Sir Jules Thorn Charitable Trust and the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnologia Francisco Jose de Caldas (COLCIENCIAS) (grant 1215-04-14364).

I thank M. A. Miles (LSH&T, United Kingdom) and Claudia M. E. Romero-Vivas (Uninorte, Colombia) for helpful advice.
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