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Dengue Virus Protein Recognition by Virus-Specific Murine CD8+ Cytotoxic T Lymphocytes

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The identification of the protein targets for dengue virus-specific T lymphocytes may be useful for planning the development of subunit vaccines against dengue. We studied the recognition by murine dengue virus-specific major histocompatibility complex class I-restricted, CD8+ cytotoxic T lymphocytes (CTL) of dengue virus proteins using recombinant vaccinia viruses containing segments of the dengue virus genome. CTL from H-2^k mice recognized a single serotype-cross-reactive epitope on the nonstructural (NS) protein NS3. CTL from H-2^b mice recognized a serotype-cross-reactive epitope that was localized to NS4a or NS4b. CTL from H-2^d mice recognized at least three epitopes: a serotype-specific epitope on one of the structural proteins, a serotype-cross-reactive epitope on NS3, and a serotype-cross-reactive epitope on NS1 or NS2a. Our findings demonstrate the limited recognition of dengue virus proteins by CTL from three inbred mouse strains and the predominance of CTL epitopes on dengue virus nonstructural proteins, particularly NS3. Since human dengue virus-specific CTL show similar patterns of recognition, these findings suggest that nonstructural proteins should be considered in designing vaccines against dengue.

Infections by the four serotypes of dengue virus cause significant morbidity in tropical areas (2). This justifies efforts to develop safe and effective vaccines against dengue. Unfortunately, the nature of protective immunity to dengue virus is not completely understood. Antibody responses to one dengue virus serotype have been associated with an increased incidence of dengue hemorrhagic fever and dengue shock syndrome during infection with another dengue virus serotype (6, 11, 28); therefore, a vaccine approach based on inducing dengue virus-specific cytotoxic T lymphocytes (CTL) might be considered. This approach has been successful in protecting mice against infection with lymphocytic choriomeningitis virus and influenza virus (14, 19). Identification of the protein targets of dengue virus-specific T cells should assist efforts to develop subunit vaccines against dengue virus (2).

We previously reported initial studies of the protein targets of dengue virus-specific human and murine CD4+ T lymphocytes (15, 26, 27). We also reported a preliminary analysis of the protein targets of human dengue virus-specific CD8+ CTL from a single subject (5). Other investigators have detected murine dengue virus-specific CTL, but the protein targets were not studied (10, 25). Work on other viral systems, such as human immunodeficiency virus, demonstrates that the epitopes recognized by murine and human CTL may overlap (13). We used recombinant vaccinia viruses containing segments of the dengue virus genome to study the protein targets and serotype specificity of dengue virus-specific CD8+ CTL in three inbred mouse strains. We observed that H-2^k CTL recognize a single epitope on nonstructural (NS) protein NS3 and H-2^b CTL recognize an epitope on one of the nonstructural proteins NS4a and NS4b. H-2^d CTL recognize several epitopes: a serotype-

cross-reactive epitope on NS3, a serotype-specific epitope on one of the structural proteins, and at lower levels, a serotype-cross-reactive epitope on nonstructural protein NS1 or NS2a.

MATERIALS AND METHODS

Cells. Target cell lines were the P815 murine mastocytoma line (H-2^d), the EL4 murine lymphoma line (H-2^b), and the L929 murine fibroblast cell line (H-2^k). L929 cells transfected with the H-2D^d protein (T4.8.3 cells) (22) were graciously provided by Carol Reiss of New York University.

Mice. Male BALB/c (H-2^d), C3H (H-2^k), and C57BL/6 (H-2^b) mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and were used between 4 and 12 weeks of age.

Viruses. Mouse-adapted dengue virus type 2 (strain New Guinea C) and dengue virus type 4 (strain 814669) were generously provided as mouse brain homogenates by Jack McCown, Walter Reed Army Institute of Research. Tissue culture-adapted dengue virus type 2 (strain New Guinea C) was kindly provided by W. Brandt, Walter Reed Army Institute of Research, and was propagated in C6/36 cells (16).

Recombinant vaccinia viruses. The recombinant viruses used in this study and the dengue virus genome segments they contain are shown in Table 1. Recombinant vaccinia viruses were constructed by insertion of dengue virus genome segments from dengue virus type 2 (strain S1) or dengue virus type 4 (strain 814669) into the pSC11 vaccinia virus intermediate vector (7) under control of the vaccinia virus P7.5 early-late promoter (3, 4, 8, 9, 30).

Generation of secondary CTL. Immunization of mice and preparation of spleen cells were performed as previously described (27). Spleen cells (3 × 10^7) were incubated in 10 ml of RPMI 1640 supplemented with 2-mercaptoethanol (50 μM) and 10% fetal bovine serum, with 0.5 ml of dengue virus (approximately 5 × 10^6 PFU). Spleen cells from mice

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TABLE 1. Recombinant vaccinia viruses expressing dengue virus genome segments

Virus	Dengue virus origin[a]	Protein sequence	Recognition by CTL from mouse line[b]:		
			H-2^k	H-2^b	H-2^d
Dengue Recombinants[c]		C-pre-M-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5			
vvD2:C-E	D2V	c-pre-M-E[d]	−	−	+*
vvD2:NS1-NS2b	D2V	e-NS1-NS2a-NS2b[e]	−	−	+§
vvD4:C-NS2a	D4V	C-pre-M-E-NS1-NS2a	−	−	+*
vvD4:E	D4V	E	−	−	−
vvD4:NS1-NS2a	D4V	NS1-NS2a	−	−	+§
vvD4:NS1-NS4b	D4V	NS1-NS2a-NS2b-NS3-NS4a-NS4b	+§	+§	+§
vvD4:NS3	D4V	NS3	+§	−	+§

[a] D2V, dengue virus type 2 strain S1; D4V, dengue virus type 4 strain 814669.

[b] Data are based on the results shown in Fig. 2 and 4 and Table 2. +* and +§, recognition by serotype-specific and serotype-cross-reactive CTL, respectively. −, no recognition.

[c] Constructed as described in Materials and Methods.

[d] A shortened C protein beginning with amino acid 41 (3).

[e] The C-terminal 39 amino acids of E, all of NS1 and NS2a, and the N-terminal 84% of NS2b (8).

immunized with dengue virus type 2 were incubated with dengue virus type 2, and spleen cells from mice immunized with dengue virus type 4 were incubated with dengue virus type 4.

Cytotoxicity assays. P815 and EL4 target cells (0.5 × 10^6 to 1 × 10^6) were incubated with dengue virus or vaccinia viruses for 2 h and then incubated overnight at 37°C. Target cells were labelled with 250 μCi of Na^51CrO_4 in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum for 1 h and then seeded in 96-well U-bottom plates at 0.5 × 10^4 to 1 × 10^4 per well.

L929 and T4.8.3 target cells (10^6) were incubated with dengue virus or vaccinia viruses for 2 h. Cells were labelled with 250 μCi of Na^51CrO_4 simultaneously with or immediately after infection. Cells were then seeded in 96-well flat-bottom plates at 0.5 × 10^4 to 1 × 10^4 per well and incubated overnight at 37°C. Wells were then washed, and fresh medium (0.1 ml per well) was added.

Secondary CTL were added to plates at various effector/target ratios, and the plates were incubated for 4 h at 37°C. The supernatant fluids were collected with a Titertek harvester, and ^51Cr release was measured in a gamma counter. Maximum ^51Cr release was determined from wells containing target cells and Renex (1:40); minimum ^51Cr release was determined from wells containing target cells and medium only. Percent specific lysis was calculated as (experimental ^51Cr release − minimum ^51Cr release)/(maximum ^51Cr release − minimum ^51Cr release) × 100.

Assays were performed in triplicate or quadruplicate wells; the standard errors of the means of samples did not exceed 10%. Minimum ^51Cr release did not exceed 25% and was usually less than 15% of the maximum ^51Cr release.

Antibody and complement depletion of responding cells. Anti-Thy1.2 and anti-L3T4 antibodies were obtained from Becton Dickinson (Mountain View, Calif.). Anti-Lyt2.1 antibody was obtained from Cedarlane (Hornby, Ontario, Canada). Antibodies were used at a concentration of 1:50 (anti-Thy1.2) or 1:20. Depletions were performed as previously described (27).

Cold-target inhibition assays. Additional L929 target cells (5 × 10^6) were incubated with dengue virus or vaccinia viruses for 2 h without radioisotope and then incubated overnight in 25-cm^2 flasks at 37°C. Cells were detached with EDTA and added to wells containing ^51Cr-labelled L929 cells

to achieve the indicated ratio of unlabelled (cold) to labelled (hot) target cells prior to the addition of effector cells.

RESULTS

Murine dengue virus-specific cytolytic activity is mediated by MHC class I-restricted CD8^+ CTL. Spleen cells from dengue virus-immunized BALB/c, C3H, and C57BL/6 mice incubated in vitro with dengue virus lysed dengue-infected histocompatible target cells (see below). Depletion of effector cell populations by treatment with antibody plus complement demonstrated that the dengue virus-specific cytotoxic cells are CD3^+ CD4^− CD8^+ T lymphocytes (data not shown).

To confirm that these dengue virus-specific CTL were major histocompatibility complex (MHC) class I restricted, we tested the activity of BALB/c CTL against T4.8.3 cells, which express the H-2D^d protein. BALB/c CTL lysed dengue virus type 2-infected T4.8.3 cells but not L929 cells, which express only H-2^k antigens (Fig. 1). This demonstrates
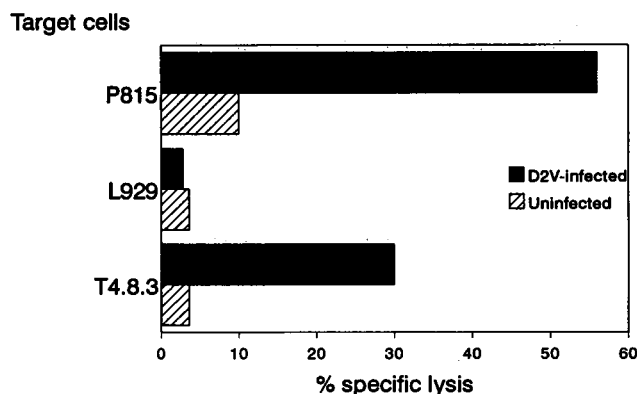
Target cells

FIG. 1. Recognition of H-2-transfected L cells by dengue virus-specific H-2^d CTL. Effector cells were spleen cells from dengue virus type 2-immunized BALB/c (H-2^d) mice, cultured as described in Materials and Methods. Target cells were dengue virus type 2-infected or uninfected P815 cells (H-2^d), L929 cells (H-2^k), or T4.8.3 cells (H-2^d-transfected, H-2^k). The effector/target ratio was 50:1.
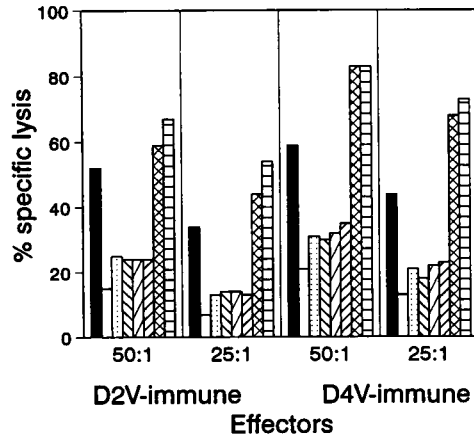
FIG. 2. Recognition by H-2^k CTL of recombinant vaccinia viruses expressing dengue virus proteins. Effector cells were spleen cells from dengue virus type 2- or type 4-immunized C3H mice, cultured as described in Materials and Methods. Target cells were L929 cells that were infected with dengue virus type 2 (D2V) (■), uninfected (□), infected with wild-type vaccinia virus (▤), or infected with recombinant vaccinia virus vvD2:C-E (▧), vvD2:NS1-NS2b (▨), vvD4:C-NS2a (▨), vvD4:NS1-NS4b (▨), or vvD4:NS3 (▤) (see Table 1). Effector/target ratio was 50:1 or 25:1 as indicated. D4V, dengue virus type 4.
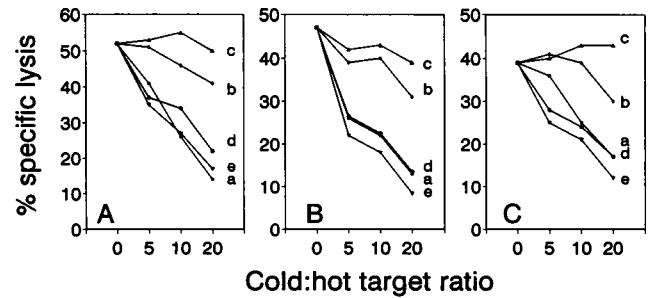

FIG. 3. Cold-target inhibition of recognition of dengue virus proteins by H-2^k CTL. Effector cells were spleen cells from dengue virus type 4-immunized C3H mice as in Fig. 2. Radiolabelled (hot) target cells were L929 cells infected with dengue virus type 2 (A), L929 cells infected with recombinant vaccinia virus vvD4:NS1-NS4b (B), or L929 cells infected with recombinant vaccinia virus vvD4:NS3 (C). Unlabelled (cold) target cells added at the indicated cold/hot target cell ratios were L929 cells that were infected with dengue virus type 2 (a), uninfected (b), infected with wild-type vaccinia virus (c), or infected with recombinant vaccinia virus vvD4:NS1-NS4b (d) or vvD4:NS3 (e). The effector/labelled target cell ratio was 30:1.

that some dengue virus-specific H-2^d CTL are restricted by the H-$2D^d$ antigen.

Dengue virus protein specificity of H-2^k CTL. To determine the specific virus proteins recognized by dengue virus-specific CTL, we constructed recombinant vaccinia viruses expressing portions of the dengue virus type 2 or dengue virus type 4 genome. Target cells were infected with the various recombinant vaccinia viruses and were incubated with secondary CTL generated from dengue virus type 2- or dengue virus type 4-immunized mice.

CTL generated from H-2^k mice immunized with either dengue virus type 2 or dengue virus type 4 showed specific lysis of dengue virus type 2-infected L929 target cells (Fig. 2). Both populations of CTL also lysed target cells infected with vvD4:NS1-NS4b or vvD4:NS3, which expressed dengue virus type 4 NS1, NS2a, NS2b, NS3, NS4a, and NS4b proteins or dengue virus type 4 NS3 protein alone, respectively (Table 1 and Fig. 2). This demonstrates that H-2^k CTL recognize an epitope on the NS3 protein of dengue virus that is cross-reactive between dengue virus type 2 and dengue virus type 4.

To determine whether NS3 is the only protein recognized by dengue virus-specific H-2^k CTL or whether other nonstructural proteins of dengue virus coded for by vvD4:NS1-NS4b were also recognized, we performed cold-target inhibition experiments. Unlabelled L929 target cells that were uninfected or infected with dengue virus type 2 or recombinant vaccinia viruses were added to ^{51}Cr-labelled L929 target cells at various cold/hot target cell ratios, and H-2^k effector cells were added. Unlabelled vvD4:NS3-infected target cells inhibited the lysis of dengue virus type 2-infected or vvD4:NS1-NS4b-infected target cells to the same degree as the unlabelled homologous target cells (Fig. 3). This indicates that NS3 protein is the major target for dengue virus-specific H-2^k CTL.

Dengue virus protein specificity of H-2^b CTL. To determine the dengue virus proteins recognized by H-2^b CTL, we

performed similar experiments using C57BL/6 mice and EL4 target cells. CTL generated from dengue virus type 2- or dengue virus type 4-immune mice showed specific lysis of dengue virus type 2-infected targets and targets infected with vvD4:NS1-NS4b, which expressed dengue virus type 4 nonstructural proteins NS1 through NS4b (Table 1 and Fig. 4). This demonstrates that H-2^b CTL recognize one or more serotype-cross-reactive epitopes on the dengue virus nonstructural proteins. H-2^b CTL did not lyse target cells infected with vvD2:NS1-NS2b, which expresses dengue virus type 2 NS1, NS2a, and 84% of NS2b, or vvD4:NS3,
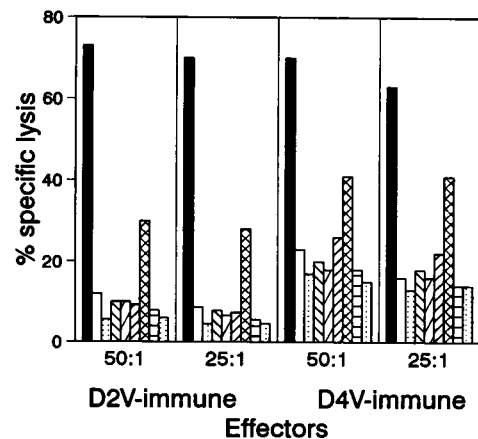

FIG. 4. Recognition by H-2^b CTL of recombinant vaccinia viruses expressing dengue virus proteins. Effector cells were spleen cells from dengue virus type 2- or dengue virus type 4-immunized C57BL/6 mice, cultured as described in Materials and Methods. Target cells were EL4 cells that were infected with dengue virus type 2 (D2V) (■), uninfected (□), infected with wild-type vaccinia virus (▤), or infected with recombinant vaccinia virus vvD2:C-E (▧), vvD2:NS1-NS2b (▨), vvD4:C-NS2a (▨), vvD4:NS1-NS4b (▨), vvD4:NS1-NS2a (▤), or vvD4:NS3 (▤) (see Table 1). Effector/target ratio was 50:1 or 25:1 as indicated. D4V, dengue virus type 4.

TABLE 2. Protein specificity of dengue virus-specific
BALB/c CTL[a]

P815 target cells[b]	% Specific lysis for the following effector cells and effector/target ratio[c]:			
	D2V		D4V	
	50:1	25:1	50:1	25:1
Expt 1				
D2V-infected	<u>75</u>	<u>65</u>	<u>83</u>	<u>78</u>
Uninfected	36	23	37	29
Vaccinia virus control	30	18	30	20
vvD2:C-E	<u>49</u>	<u>35</u>	38	27
vvD2:NS1-NS2b	41	26	<u>49</u>	<u>37</u>
vvD4:C-NS2a	38	20	<u>69</u>	<u>62</u>
vvD4:E	38	22	33	23
vvD4:NS1-NS2a	37	20	<u>52</u>	<u>41</u>
vvD4:NS1-NS4b	<u>58</u>	<u>34</u>	60	<u>55</u>
Expt 2				
D2V-infected	ND	ND	<u>66</u>	<u>40</u>
Uninfected	ND	ND	28	15
Vaccinia virus control	ND	ND	27	17
vvD4:C-NS2a	ND	ND	<u>43</u>	<u>29</u>
vvD4:E	ND	ND	23	12
vvD4:NS3	ND	ND	<u>45</u>	<u>26</u>

[a] D2V and D4V, dengue virus type 2 and type 4, respectively.

[b] P815 target cells were uninfected or infected with dengue virus type 2 or wild-type or recombinant vaccinia viruses.

[c] Spleen cells from mice immunized with dengue virus type 2 or 4 were cultured as described in Materials and Methods. Also, see Materials and Methods for the method of calculation of percent specific lysis. Values that are >3 standard deviations higher than the corresponding values for uninfected target cells and target cells infected with wild-type vaccinia virus are underlined. ND, not determined.

which expresses dengue virus type 4 NS3 protein (Fig. 4), demonstrating that the serotype-cross-reactive epitope(s) lies on the dengue virus nonstructural protein NS4a or NS4b or the C-terminal 16% of NS2b.

Dengue virus protein specificity of H-2^d CTL. We also determined the dengue virus protein specificity of H-2^d CTL, using BALB/c mice and P815 target cells. As was observed with H-2^k and H-2^b CTL, CTL generated from dengue virus type 2- or dengue virus type 4-immunized H-2^d mice showed specific lysis of dengue virus type 2-infected target cells (Table 2), demonstrating a serotype-cross-reactive CTL response. Both populations of CTL also lysed target cells infected with vvD4:NS1-NS4b. As was seen with H-2^k CTL, H-2^d CTL did recognize target cells infected with vvD4:NS3 (Table 2 [experiment 2]), demonstrating that H-2^d CTL recognize an epitope on the NS3 protein.

In addition, there were important differences in specificity between the dengue virus type 2- and dengue virus type 4-immune H-2^d CTL populations. CTL from dengue virus type 2-immunized H-2^d mice lysed target cells infected with vvD2:C-E but not target cells infected with vvD4:C-NS2a, whereas the reverse was true of CTL from dengue virus type 4-immunized H-2^d mice (Table 2). This indicates that H-2^d CTL recognize a serotype-specific epitope on one of the dengue virus structural proteins. The failure of dengue virus type 4-specific H-2^d CTL to recognize target cells infected with vvD4:E (Table 2) suggests that one of the other structural proteins, C or pre-M, contains a CTL epitope.

CTL from dengue virus type 4-immunized H-2^d mice but not CTL from dengue virus type 2-immunized H-2^d mice showed lower but still significant levels of lysis of target cells infected with vvD4:NS1-NS2a and vvD2:NS1-NS2b (Table

2). This suggests that H-2^d CTL recognize another serotype-cross-reactive epitope on dengue virus protein NS1 or NS2a. Lysis of target cells infected with vvD4:C-NS2a was always significantly higher than lysis of target cells infected with vvD4:NS1-NS2a, suggesting that there are CTL epitopes on several dengue virus proteins. These results are summarized in Table 1 and show that H-2^d CTL recognize several dengue virus epitopes, both serotype specific and serotype cross-reactive.

DISCUSSION

Murine dengue virus-specific MHC class I-restricted, CD8+ CTL have not been well characterized (10, 25). We have analyzed the serotype specificity and protein specificity of these CTL using recombinant vaccinia viruses expressing dengue virus type 2 or dengue virus type 4 proteins. Our results show that dengue virus-specific H-2^k CTL exclusively recognize a dengue virus type 2 and dengue virus type 4 cross-reactive epitope on the nonstructural protein, NS3. Dengue virus-specific H-2^b CTL recognize a dengue virus type 2 and dengue virus type 4 cross-reactive epitope(s) on a different nonstructural protein, probably NS4a or NS4b. Further studies using additional recombinants which express isolated dengue virus proteins will be necessary to identify which of these proteins contains the epitope(s) recognized by H-2^b CTL.

Dengue virus-specific H-2^d CTL recognized at least three dengue virus epitopes. Like H-2^k CTL, these CTL recognized a dengue virus type 2 and dengue virus type 4 cross-reactive epitope on NS3. In addition, H-2^d CTL recognize a serotype-specific CTL epitope on one of the structural proteins of dengue virus, probably C or pre-M. CTL from dengue virus type 4-immune H-2^d mice also recognized a dengue virus type 2 and dengue virus type 4 cross-reactive epitope on the NS1 or NS2a protein, although at levels lower than the levels of recognition of the other CTL epitopes. We attribute the failure of CTL from dengue virus type 2-immunized H-2^d mice to lyse target cells expressing NS1 and NS2a proteins to a less efficient stimulation of NS1- and NS2a-specific CTL in that culture.

We found that CD8+ CTL from each mouse strain recognized a very limited number of dengue virus epitopes, as few as one to three CTL epitopes on the entire genome, which encodes 3,386 amino acids (21, 29). Our preliminary studies suggest that this holds for human dengue virus-specific CD8+ CTL as well (5), although human and murine dengue virus-specific CD4+ T cells appear to recognize a larger number of epitopes (15, 26). Similarly, for Kunjin virus (another flavivirus), CD8+ CTL from mice of four of the five H-2 haplotypes studied recognized only a single immuno-dominant epitope (12). In contrast, murine influenza virus-specific CTL recognized three or four virus proteins in the several mouse strains tested (1). The limited recognition of flaviviruses by CTL suggests that flaviviruses may have mutations in protein sequences that are efficiently presented with MHC antigens.

We found a predominance of CTL epitopes on nonstructural proteins, as has been observed with other viruses such as herpes simplex virus (23). In particular, the nonstructural protein, NS3, was important for recognition by CD8+ CTL. CTL from two of the three mouse strains tested recognized an epitope on NS3. NS3 has been shown to be a major site of human CD4+ and CD8+ T-cell epitopes (5, 18, 20). The major Kunjin virus epitope for murine H-2^k, H-2^d, and H-2^q CD8+ CTL was also localized to a region containing the last

34 amino acids of NS3 and the first 65 amino acids of NS4a (12). It is not known whether the intracellular trafficking of NS3 is different from that of other flavivirus proteins or whether mutations of NS3 which might escape presentation with MHC antigens are lethal for the virus. However, the reproducibility of recognition of NS3 by T cells in humans and mice suggests that NS3 may be worth considering for experimental subunit vaccines against dengue.

Our findings may help to explain the protective effects of some candidate subunit vaccines against lethal intracerebral infection of mice with dengue virus. Several studies have reported protection of mice after immunization with recombinant dengue virus proteins in the absence of detectable antibodies to dengue virus. These preparations have included recombinant vaccinia viruses expressing C, pre-M, and E proteins or M protein alone (3, 4). Our study suggests that immunization of BALB/c mice with vaccines containing structural proteins might have induced dengue virus-specific CTL, which could have contributed to the protective immunity.

Vaccination designed to induce CTL responses has protected against lethal infection with lymphocytic choriomeningitis virus (14), but it is impossible to predict whether this approach will be beneficial or harmful in dengue virus infection of humans. CTL induced by vaccination worsen the clinical manifestations of lymphocytic choriomeningitis virus infection under certain conditions (24). We recently found evidence that dengue hemorrhagic fever and dengue shock syndrome are associated with high levels of activation of CD4$^+$ and CD8$^+$ T cells as measured by serum soluble-CD4, soluble-CD8, and soluble-interleukin-2 receptor levels (17). The majority of CTL responses we observed in this study, and in our studies of the human CTL responses to dengue virus (5, 15), were directed at serotype-cross-reactive epitopes and could be activated in secondary dengue virus infections. An immunopathological role for cross-reactive T cells in dengue is difficult to prove in the murine model because dengue virus infections do not cause hemorrhagic fever in mice.

Effective vaccines should presumably generate both appropriate humoral immunity and appropriate cellular immunity to provide the best balance of protective immune responses. Further studies on the CTL responses to dengue virus should provide additional information to guide development of safe and effective vaccines.

ACKNOWLEDGMENTS

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