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Multiple Specificities in the Murine CD4$^+$ and CD8$^+$ T-Cell Response to Dengue Virus

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The target epitopes, serotype specificity, and cytolytic function of dengue virus-specific T cells may influence their theoretical roles in protection against secondary infection as well as the immunopathogenesis of dengue hemorrhagic fever. To study these factors in an experimental system, we isolated dengue virus-specific CD4$^+$ and CD8$^+$ T-cell clones from dengue-2 virus-immunized BALB/c mice. The T-cell response to dengue virus in this mouse strain was heterogeneous; we identified at least five different CD4$^+$ phenotypes and six different CD8$^+$ phenotypes. Individual T-cell clones recognized epitopes on the dengue virus pre-M, E, NS1/NS2A, and NS3 proteins and were restricted by the I-A^d, I-E^d, L^d, and K^d antigens. Both serotype-specific and serotype-cross-reactive clones were isolated in the CD4$^+$ and CD8$^+$ subsets; among CD8$^+$ clones, those that recognized the dengue virus structural proteins were serotype specific whereas those that recognized the nonstructural proteins were serotype cross-reactive. All of the CD8$^+$ and one of five CD4$^+$ clones lysed dengue virus-infected target cells. Using synthetic peptides, we identified an L^d-restricted epitope on the E protein (residues 331 to 339, SPCKIPFEI) and a K^d-restricted epitope on the NS3 protein (residues 296 to 310, ARGYISTRVEM GEAA). These data parallel previous findings of studies using human dengue virus-specific T-cell clones. This experimental mouse system may be useful for studying the role of the virus serotype and HLA haplotype on T-cell responses after primary dengue virus infection.

Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), the severe manifestation of infection by the flavivirus dengue virus, is a significant cause of morbidity and mortality in tropical regions of the world (20, 36). Recently DHF/DSS has occurred in increasing numbers and over a wider geographic distribution. Preexisting immunity to one of the four dengue virus serotypes has been shown to increase the risk of DHF/DSS during infection with a second dengue virus serotype (5, 17, 45). Antibody-dependent enhancement of dengue virus infection of monocytic cells has been proposed as one explanation for this observation (18, 19, 37). We and others have also proposed that serotype-cross-reactive dengue virus-specific T cells play a role in the development of the plasma leakage syndrome characteristic of DHF/DSS by releasing vasoactive cytokines and lysing dengue virus-infected monocytes (23, 29).

We have previously reported that DHF/DSS is characterized by marked T-cell activation, as measured by serum levels of the cytokines interleukin-2 (IL-2) and gamma interferon as well as the soluble forms of CD4, CD8, and the α chain of the IL-2 receptor (25). Our laboratory has also identified some of the molecular targets and in vitro functions of human dengue virus-specific CD4$^+$ and CD8$^+$ T cells in bulk culture populations and at the clonal level (4, 14, 16, 22, 24, 26–28, 31, 32).

Murine T-cell responses have been a useful guide to understanding the human T-cell responses to human immunodeficiency virus and hepatitis C virus (7, 21, 47, 48). We previously described the serotype and protein specificity of murine dengue virus-specific CD4$^+$ and CD8$^+$ T cells in bulk culture (42–44). We found both serotype-specific and serotype-cross-reactive T-cell responses in the CD4$^+$ and CD8$^+$ populations, similar to the observations for human dengue virus-specific T cells. We also found murine CD8$^+$ T-cell responses to the dengue virus nonstructural protein NS3, which has been a target for human CD4$^+$ and CD8$^+$ cytotoxic T lymphocytes (CTL).

In this study, we extend the previous observations on dengue virus-specific murine T cells by isolating CD4$^+$ and CD8$^+$ T-cell clones, and we describe the serotype and protein specificity as well as the in vitro cytolytic functions of these T-cell clones. For both CD4$^+$ and CD8$^+$ populations, some individual clones were serotype specific whereas others were serotype cross-reactive. We identified T-cell clones specific for at least 4 of the 10 proteins of dengue virus type 2 (dengue-2 virus). All of the CD8$^+$ T-cell clones and one of five CD4$^+$ T-cell clones lysed dengue virus-infected cells in vitro. Two CD8$^+$ CTL epitopes, one on the E protein (amino acids 331 to 339) and one on the NS3 protein (amino acids 296 to 310), were mapped by using synthetic peptides.

MATERIALS AND METHODS

Cells. Target cells were the A20 murine lymphoma line (*H-2^d*), the P815 murine mastocytoma line (*H-2^d*), the L929 murine fibroblast cell line (*H-2^k*), and L929 cells transfected with L^d (T1.1.1), D^d (T4.8.3; both from Carol Reiss, New York University) (34), or K^d (L-K^d-172; from Jack Bennink, National Institutes of Health) (10).

Mice. Male BALB/c (*H-2^d*) mice were obtained from the Charles River Breeding Laboratories (Wilmington, Mass.). Female B10.RDD (*I-A^{d+}*) mice were generously provided by Chella David, Mayo Clinic (15). Mice were used between 4 and 12 weeks of age.

Viruses and viral antigens. Mouse-adapted dengue-1 virus (strain Hawaii), dengue-2 virus (strain New Guinea C), dengue-3 virus (strain PR6), and dengue-4 virus (strain 814669) were generously provided as mouse brain homogenates by Jack McCown, Walter Reed Army Institute of Research. Tissue culture-adapted dengue-2 virus (strain New Guinea C) was kindly provided by Walter Brandt, Walter Reed Army Institute of Research, and was propagated in C6/36 cells.

Recombinant vaccinia viruses expressing proteins of dengue-2 or dengue-4 virus under control of the vaccinia virus P7.5 early-late promoter were provided

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by C.-J. Lai, National Institute of Allergy and Infectious Diseases (2, 3, 11, 12, 42, 49). The dengue virus proteins expressed by these recombinant vaccinia viruses are designated on the basis of the dengue virus serotype that served as the source for cloning and the genome segment included. The range of proteins expressed is listed on the basis of the order of proteins in the flavivirus polyprotein, C–prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, where prM is the precursor for the membrane protein M (pre-M).

Vero cell-derived dengue virus antigens were prepared as previously described (26, 27, 44). Immunoaffinity-purified dengue-2 virus NS1 protein was provided by Jacob Schlesinger, Rochester General Hospital (46). Purified dengue-2 virus E, NS3, and NS5 proteins were provided by Margo Brinton, Georgia State University (23).

Synthetic peptides were prepared by 9-fluorenylmethoxycarbonyl chemistry either in our laboratory by using the RaMPS multiple-peptide synthesizer (DuPont) or in the Peptide Core facility at the University of Massachusetts Medical Center by using a Symphony automated peptide synthesizer (Rainin Instruments).

Generation of dengue virus-specific T-cell clones. Immunization of mice and preparation of spleen cells were performed as previously described (42, 44). Spleen cells (3×10^7) were incubated in 10 ml of RPMI 1640 supplemented with 2-mercaptoethanol (50 μM) and 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah) with 0.1 ml of Vero cell-derived dengue-2 virus antigen or 0.5 ml of dengue-2 virus (approximately 5×10^6 PFU). Proliferating cells were restimulated every 1 to 2 weeks by culturing them in the presence of 10% rat lectin-free T-cell growth factor (TCGF) as a source of IL-2 with either 3×10^7 γ-irradiated syngeneic nonimmune spleen cells plus 1:100-diluted Vero cell-derived dengue-2 virus antigen (for $CD4^+$ clones) or 2×10^6 γ-irradiated, dengue-2 virus-infected, major histocompatibility complex (MHC)-matched target cells (for $CD8^+$ clones).

Cloning of dengue virus-specific $CD4^+$ T cells was performed by limiting dilution in 96-well flat-bottom plates with 10^6 γ-irradiated syngeneic nonimmune spleen cells per well in the presence of 1:100-diluted Vero cell-derived dengue-2 virus antigen and 10% rat TCGF.

Cloning of dengue virus-specific $CD8^+$ T cells was performed by limiting dilution in 96-well U-bottom plates with 10^4 γ-irradiated, dengue-2 virus-infected, MHC-matched target cells per well in the presence of 10% rat TCGF.

Proliferation assays. Proliferation assays using $CD4^+$ T-cell clones were performed at least 10 days after stimulation with antigen. T cells (2×10^4) were incubated with γ-irradiated syngeneic (except as noted) spleen cells (2×10^5) and various concentrations of antigen in 0.2 ml of complete medium supplemented with 0.5% rat TCGF in each well of 96-well U-bottom plates. Plates were incubated for 72 h at 37°C. [^{3}H]thymidine (1.25 μCi per well) was added for the final 6 h of incubation. Cells were harvested onto glass filter paper with a semiautomatic cell harvester (Skatron, Inc., Sterling, Va.), and the [^{3}H]thymidine incorporation was measured in a Betaplate liquid scintillation counter (LKB Wallac Oy, Turku, Finland).

Cytotoxicity assays. Target cells were infected with dengue virus or vaccinia viruses for 2 h and then incubated overnight at 37°C. A20 target cells were incubated overnight with Vero cell-derived antigens at a dilution of 1:20. Target cells were labeled with 250 μCi of $Na_2{}^{51}CrO_4$ in 0.2 ml of medium for 1 h and then seeded in 96-well U-bottom plates at 0.5×10^4 to 1×10^4 cells per well. For experiments using synthetic peptides, peptides were added at various concentrations directly to wells containing target cells.

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

Assays were performed in triplicate wells. The standard error of the mean of samples did not exceed 10%. Minimum ^{51}Cr release was generally less than 20% of the maximum ^{51}Cr release.

RESULTS

Serotype specificity of dengue virus-specific $CD4^+$ clones. We stimulated dengue-2 virus-immune BALB/c splenocytes with inactivated dengue virus antigens and isolated T-cell clones by limiting dilution, as described for the isolation of dengue virus-specific human $CD4^+$ T-cell clones (14, 16, 27, 28). Five T-cell clones were isolated. By fluorescent antibody staining, all expressed the $Thy1^+$ $CD4^+$ $CD8^-$ phenotype. Specificity of the clones for dengue virus was analyzed by proliferation assay using inactivated dengue virus antigens (Table 1). All of the clones showed proliferative responses to dengue-2 virus antigen. Three of the five clones were dengue-2 virus specific, showing no proliferative response to antigens of the other three serotypes. One clone showed a low but reproducible proliferative response to dengue-4 virus antigen. The fifth clone was broadly cross-reactive to all four dengue virus serotypes and consistently showed the highest proliferative response to dengue-4 virus antigen.

TABLE 1. Proliferative responses of $CD4^+$ T-cell clones to dengue virus antigens

Clone	Stimulation index[a] to indicated antigen			
	D1	D2	D3	D4
2A1	0.4	2.5	0.6	0.5
2A2	0.5	136	0.4	4.3
2A3	1.3	6.4	1.4	1.3
2A4	5.3	11.5	19.7	50.5
2A5[b]	0.8	32	1.2	1.1

[a] Stimulation index = (cpm of cells incubated with indicated antigen)/(cpm of cells incubated with control antigen). Data are listed as a composite of results from four different experiments involving the five T-cell clones. Experimental conditions were identical except as noted. D1 to D4 indicate antigens from dengue-1 to dengue-4 viruses. Underlined values are significantly higher than those for controls.

[b] Infectious virus (C6/36 culture supernatant) was used as the source of antigen; culture supernatant from uninfected C6/36 cells was used as the control antigen.

Protein specificity and MHC restriction of dengue virus-specific $CD4^+$ clones. We next analyzed the proliferative response of these T-cell clones to purified E, NS1, NS3, and NS5 proteins of dengue-2 virus. No proliferative responses by any of the five clones were observed in assays using the E, NS3, and NS5 proteins (data not shown). The dengue-2 virus-specific clone 2A3 showed a concentration-dependent proliferation to the purified dengue-2 virus NS1 protein (Table 2), whereas none of the other four clones proliferated after incubation with NS1 protein.

To determine the class II restriction of these $CD4^+$ clones, we analyzed the proliferation of the clones in the presence of irradiated spleen cells from BALB/c ($I\text{-}A^{d+}$ $I\text{-}E^{d+}$) and B10.RDD ($I\text{-}A^{d+}$ $I\text{-}E^{d-}$) mice. The dengue-2 virus-specific clone 2A1 proliferated in the presence of dengue-2 virus antigen and spleen cells from both mouse strains (Table 3), indicating that this clone recognizes dengue virus antigen in the context of $I\text{-}A^d$, whereas the other four clones proliferated only in the presence of BALB/c spleen cells, indicating that these clones recognize dengue antigen in the context of $I\text{-}E^d$.

Cytotoxic activity of dengue virus-specific $CD4^+$ clones. We tested these clones for cytotoxic activity by incubating them with A20 target cells, which express $H\text{-}2^d$ class II antigens, in a 4-h chromium release assay. Only clone 2A2 lysed dengue-2 virus antigen-pulsed target cells (Table 4). In a separate experiment, clone 2A2 also lysed dengue-2 virus-infected A20 cells but was unable to lyse dengue-2 virus-infected P815 cells,

TABLE 2. Recognition of purified NS1 protein by $CD4^+$ T-cell clone 2A3

Dilution	[^{3}H]thymidine incorporation by clone 2A3 (cpm)	
	Dengue-2 virus NS1 protein	Control
1:10	3,669	106
1:50	3,137	130
1:250	816	62
1:1,250	102	55

TABLE 3. MHC restriction of proliferative responses of dengue virus-specific CD4$^+$ T-cell clones

Clone	Stimulation index[a] obtained with stimulator cells from indicated mouse strain (*H-2^d* class II allele[s])	
	BALB/c (*I-A^d*, *I-E^d*)	B10.RDD (*I-A^d*)
2A1	16	9.3
2A2	4.3	1.4
2A3	61	1.3
2A4	25	1.3
2A5	6.2	1.1

[a] Stimulation index = (cpm of cells incubated with dengue-2 virus antigen)/(cpm of cells incubated with control antigen). Underlined values are significantly higher than those for controls.

which do not express class II MHC antigens (data not shown). These data indicate that clone 2A2 is a cytotoxic CD4$^+$ clone.

Serotype and protein specificity of dengue virus-specific CD8$^+$ clones. In vitro stimulation with inactivated antigens has been shown to favor the growth of CD4$^+$ T cells (39). To select for the growth of CD8$^+$ T cells, we stimulated dengue-2 virus-immune BALB/c splenocytes with infectious dengue-2 virus and then maintained the cells by stimulation with γ-irradiated, dengue-2 virus-infected P815 cells in the presence of IL-2. Cells were then cloned by limiting dilution. Clones were screened for specific cytotoxic activity against dengue-2 virus-infected P815 cells. Representative results are shown in Table 5. None of the clones isolated lysed uninfected P815 cells (Table 5 and data not shown). All of the clones tested expressed the Thy1$^+$ CD4$^-$ CD8$^+$ phenotype. The serotype and protein specificity of the clones was determined by testing for lysis of P815 target cells infected with recombinant vaccinia viruses expressing dengue virus proteins.

We identified at least six different types of dengue virus-specific CTL clones. Three of these CTL clones recognized target cells infected with vvD2:C-prM-E, which expresses the dengue-2 virus structural proteins, as exemplified in Table 5 by clones 2D13, 2E-56, and 2E-60. Clone 2D13 recognized target cells infected with vvD2:E, which expresses only the dengue-2 virus E protein (Table 5, experiment 1), as did clone 2E-60 (Table 5, experiment 5), although the specific epitopes recognized differed (see below). Clone 2E-56 recognized target cells infected with vvD2:prM, which expresses only the pre-M protein (Table 5, experiment 5). None of these clones recognized the dengue-4 virus structural proteins expressed by recombinant vaccinia virus vvD4:C-prM-E-NS1-NS2A (data not shown), suggesting that these CTL clones were serotype specific.

Two different CTL recognized dengue virus nonstructural proteins, as exemplified in Table 5 by clones 2D42, 2D65, and 2E-67. Clone 2D42 recognized target cells infected with vvD2:NS3, which expresses the dengue-2 virus NS3 protein (Table 5, experiment 1), or vvD4:NS3 (Table 5, experiment 2), which expresses the dengue-4 virus NS3 protein. Clone 2D65 also recognized the dengue-2 and dengue-4 virus NS3 protein (Table 5, experiment 2). Clone 2E-67 recognized target cells infected with vvD2:NS1-NS2A-NS2B, which expresses the dengue-2 virus NS1 and NS2A proteins and most of the NS2B protein, as well as target cells infected with vvD4:NS1-NS2A, which expresses the dengue-4 virus NS1 and NS2A proteins (Table 5, experiment 4). These results indicate that these CTL clones specific for nonstructural proteins were serotype cross-reactive.

Some CTL clones recognized none of the available recombinant vaccinia viruses, although they did recognize dengue-2 virus-infected target cells, as exemplified by clones 2D7 (Table 5, experiment 3) and 2E-8 (Table 5, experiment 4). Thus, there must be at least a sixth CTL specificity. From analyses using the available recombinant vaccinia viruses, this epitope may lie on the N terminus of C, on the C terminus of NS2B, or on the NS4A, NS4B, or NS5 protein.

MHC restriction of recognition by CD8$^+$ CTL clones. We studied the MHC restriction of recognition of some of the CTL clones, using target cells sharing only one of the *H-2^d* class I genes. In these experiments, we used clone 2D13, which recognized the dengue-2 virus E protein (Table 5), and clones 2D42, 2D63, and 2D65, which all recognized the dengue-2 virus NS3 protein (Table 5 and data not shown). Clone 2D13 recognized dengue-2 virus-infected target cells which expressed the L^d antigen, whereas clones 2D42, 2D63, and 2D65 recognized dengue-2 virus-infected target cells which expressed the K^d antigen (Table 6).

Mapping of CTL epitopes on E and NS3 proteins. We used synthetic peptides to identify the specific epitopes recognized on the E and NS3 proteins. To map the epitope on the NS3 protein, we first tested for recognition of recombinant vaccinia viruses expressing portions of the dengue-4 virus NS3 protein; this analysis indicated that the epitope was located in the middle one-third of the NS3 protein (data not shown). We then prepared 22 overlapping 15-residue synthetic peptides covering the region of interest excluding peptides that were not highly conserved between dengue-2 and dengue-4 viruses. We then tested these peptides for the ability to sensitize uninfected P815 target cells for recognition by the K^d-restricted, NS3-specific CTL clones 2D42 and 2D65. Both CTL clones recognized target cells incubated with the overlapping peptides 10 and 11 (Table 7). Peptide 10, which was recognized to a greater extent, represents amino acids 296 to 310 of the NS3 protein, ARGYISTRVEMGEAA, which is completely conserved in dengue-2 and dengue-4 viruses. Four other CTL clones, including clone 2D63, also recognized this epitope (data not shown).

To map the epitope on the E protein recognized by clone 2D13, we identified all 9-mer peptides containing a proline at position 2, which is an anchor residue for binding to the L^d antigen (13). Of 19 peptides which met this requirement, we selected 7 peptides for synthesis after preliminary studies which showed an absence of recognition of a panel of E peptides obtained from John Roehrig, Centers for Disease Control and Prevention, Fort Collins, Colo. (data not shown). When these seven peptides were tested for the ability to sensitize target cells for lysis by clone 2D13, only one peptide, E(331-339), representing amino acids 331 to 339 of the E protein (SPCKIPFEI), was recognized (Table 8). At least 20 other CTL clones which also recognized this E(331-339) epitope were isolated. However, clone 2E-60, which recognized the dengue-2 virus E protein, did not recognize peptide E(331-339) or any of the peptides listed in Table 8 (data not

TABLE 4. Cytolytic activity of CD4$^+$ clone 2A2

Effector/target ratio	% Specific lysis of A20 target cells pulsed with[a]:					
	Antigen from:					No antigen
	D1	D2	D3	D4	Control	
10:1	0	57	0	2	<0	1
5:1	1	46	0	1	0	0

[a] A20 target cells were incubated overnight with the indicated antigen; antigens were prepared as sonic extracts of Vero cells infected with dengue-1 virus (D1), dengue-2 virus (D2), dengue-3 virus (D3), dengue-4 virus (D4), or no virus (control). Underlined values are significantly higher than those for controls.

TABLE 5. Recognition of dengue virus proteins by dengue virus-specific CTL clones

Effector	Effector/target ratio	% Specific lysis of P815 target cells infected with[a]:									
		Dengue-2 virus	No virus	Vaccinia virus control	vvD2:C-prM-E	vvD2:prM	vvD2:E	vvD2:NS1-NS2A-NS2B	vvD2:NS3	vvD4:NS1-NS2A	vvD4:NS3
Expt 1											
2D13	2:1	35	0	0	36	NT	25	2	<0	NT	NT
2D42	2:1	35	5	1	1	NT	NT	2	39	NT	NT
Expt 2											
2D42	2:1	62	8	1	NT	NT	NT	NT	49	NT	48
2D65	2:1	64	3	2	NT	NT	NT	NT	53	NT	51
Expt 3											
2D7	6:1	39	4	3	4	NT	NT	1	9	NT	NT
Expt 4											
2E-8	5:1	26	<0	0	0	NT	NT	<0	<0	0	NT
2E-60	5:1	38	0	<0	22	NT	NT	3	<0	<0	NT
2E-67	5:1	34	5	3	5	NT	NT	14	2	18	NT
Expt 5											
2E-56	5:1	NT	NT	<0	41	30	<0	NT	NT	NT	NT
2E-60	5:1	NT	NT	<0	20	<0	28	NT	NT	NT	NT

[a] Underlined values are significantly higher than those for controls. NT, not tested.

shown), indicating that there is a second epitope on the E protein recognized by *H-2*^{d} CTL. Six other CTL clones recognized the dengue-2 virus structural proteins but have not been further characterized.

DISCUSSION

We isolated dengue virus-specific CD4$^+$ and CD8$^+$ T-cell clones from dengue-2 virus-immunized BALB/c (*H-2*^{d}) mice. Our findings show a surprising level of heterogeneity in the protein specificity, serotype specificity, and MHC restriction of dengue virus-specific CD4$^+$ and CD8$^+$ T cells in this mouse strain. We identified at least five distinct in vitro phenotypes of CD4$^+$ clones and six distinct phenotypes of CD8$^+$ clones, as summarized in Table 9.

Among the CD4$^+$ T-cell clones isolated after primary dengue virus infection, serotype-specific proliferative responses were more common than serotype-cross-reactive responses. However, one of five CD4$^+$ clones was broadly serotype cross-reactive, with the highest proliferative response to dengue-4 virus antigens, although the clone was derived from a dengue-2 virus-immunized animal. This pattern of T-cell response has not yet been noted in our studies of dengue virus-immune humans and supports the notion that primary dengue virus infection can induce the proliferation of a wide variety of dengue virus serotype-cross-reactive T-cell clones. Among CD8$^+$ T-cell clones, those clones which recognized structural proteins were serotype specific and those which recognized nonstructural proteins were serotype cross-reactive, consistent with the observation that the nonstructural proteins, particularly NS1, NS3, and NS5, are more highly conserved among the four dengue virus serotypes (40).

One CD4$^+$ T-cell epitope was localized to the NS1 protein. CD8$^+$ T-cell epitopes were localized to the pre-M, E (two separate epitopes), NS1/NS2A, and NS3 proteins. A sixth epitope could not be definitively mapped with the available recombinant vaccinia viruses. One of the epitopes on the E protein was mapped to residues 331 to 339 and was shown to be restricted by the H-2L^d antigen. The epitope on the NS3 protein was mapped to residues 296 to 310 and was shown to be restricted by the H-2K^d antigen.

The CD8$^+$ T-cell clones were identified on the basis of their ability to lyse dengue virus-infected target cells. We also found that one of the five CD4$^+$ clones isolated demonstrated MHC class II-restricted killing of dengue virus-infected or antigen-pulsed cells. In contrast, almost all human dengue virus-specific CD4$^+$ clones that we have studied have expressed cytolytic function in vitro (14, 16, 22, 27, 32). Our results with murine CD4$^+$ T-cell clones are similar to the findings of other investigators (9, 33); the higher frequency of cytolytic function among dengue virus-specific human CD4$^+$ T cells may reflect the importance of HLA class II antigen-positive cells in dengue virus replication in vivo (18, 19, 23, 29).

Our data on the specificity of dengue virus-specific T-cell clones correlated well, in general, with the results obtained in the analysis of BALB/c dengue virus-specific T cells at the bulk culture level (42, 44). Proliferative responses of CD4$^+$ T cells in bulk cultures were predominantly serotype specific, with a low level of serotype cross-reactivity. Our previous bulk culture analyses had shown that dengue virus-specific BALB/c CD4$^+$ T cells recognized the NS1 protein (43) and CD8$^+$ T cells recognized the NS3 and NS1/NS2A proteins as well as one (or more) of the structural proteins (42). However, the data from the analyses of dengue virus-specific T cells in bulk culture and at the clonal level disagreed on two points. First, spleen cells from dengue-4 virus-immunized BALB/c mice showed a specific proliferative response to recombinant baculovirus-expressed dengue-4 virus E protein (43), whereas none of the dengue-2 virus-induced CD4$^+$ T-cell clones proliferated in re-

TABLE 6. MHC restriction of CTL activity of dengue virus-specific CD8$^+$ T-cell clones

Target cell	H-2^d antigen(s)	Virus	% Specific lysis by indicated clone[a]			
			Expt 1		Expt 2	
			2D13	2D63	2D42	2D65
P815	K, L, D	None	<0	<0	1	<0
		D2V[b]	44	22	84	80
T4.8.3	D	D2V	1	4	NT	NT
T1.1.1	L	D2V	22	1	NT	NT
L-K^d-172	K	D2V	1	18	55	31
DAP		D2V	2	2	3	2

[a] Underlined values are significantly higher than those for controls. NT, not tested.

[b] D2V, dengue-2 virus.

TABLE 7. Recognition of NS3 peptides by $CD8^+$ CTL clones

Peptide (0.1 μM)	% Specific lysis by clone[a]:	
	2D42	2D65
None	<0	<0
1-9	<0	<0
10	77	48
11	40	12
12-22	<0	<0

[a] Underlined values are significantly higher than those for controls.

sponse to purified dengue-2 virus E protein. Second, CTL generated in bulk culture from dengue-4 virus-immunized BALB/c mice did not recognize the dengue-4 virus E protein (42), whereas many of the $CD8^+$ clones that we isolated from dengue-2 virus-immunized mice recognized the dengue-2 virus E protein. Since we studied a small number of $CD4^+$ clones, we cannot exclude sampling as the explanation for the first observation, and indeed other investigators have shown that the dengue-2 virus E protein contains helper T-cell epitopes (30, 41). However, an alternative explanation for both findings is that immunization with different dengue virus serotypes may generate different patterns of $CD4^+$ and $CD8^+$ responses. Amino acid differences between the different dengue virus serotypes might involve critical anchor residues affecting binding to specific MHC molecules. This could have important implications for the development of synthetic vaccines against dengue virus infection, because comparable responses to each of the four dengue virus serotypes cannot be assumed.

Protective immunity against lethal encephalitis has been elicited in BALB/c mice by immunization with recombinant vaccinia viruses expressing dengue virus pre-M, E, or NS1 protein (2, 8, 11, 35, 46). The mechanism of protective immunity has not been clearly defined. Although in some cases protection could be passively transferred with serum, protection has not correlated with the generation of antibodies capable of neutralizing dengue virus in vitro (2, 11, 35, 46). Our results suggest that the generation of dengue virus-specific CTL responses could provide one mechanism of protection in these studies.

The human $CD4^+$ T-cell response to dengue virus has been studied in greater detail than the $CD8^+$ T-cell response to this virus. Nonetheless, the findings presented here indicate that murine and human T-cell responses to dengue virus are qualitatively similar with regard to recognition of heterologous dengue virus serotypes and cytolytic function. Some human subjects had a higher frequency of serotype-cross-reactive T-cell clones than others (16, 27), which could be influenced by the serotype of dengue virus with which they have been infected and their HLA antigens. Most human dengue virus-specific $CD4^+$ T-cell clones have shown the Th1 phenotype, producing gamma interferon and IL-2 and lysing dengue virus-infected MHC class II-expressing cells (14a, 27). We found that one of five murine dengue virus-specific $CD4^+$ T-cell clones had cytolytic function in vitro; however, we have not detected IL-2 (or IL-4) production by these clones in vitro in a bioassay in CTLL cells (data not shown). We have hypothesized that the pattern of cytokine production, the cytolytic function, and the proportion of serotype-cross-reactive cells all might influence the immune response to infection with a second dengue virus serotype and could affect the likelihood of an immunopathological response leading to DHF/DSS.

Some investigators have reported that murine and human T cells recognize identical or overlapping epitopes in human immunodeficiency virus and hepatitis C virus (7, 21, 47, 48). Although we have found that some human and murine dengue virus-specific T cells recognize the same proteins, such as the E, NS1/NS2A, and NS3 proteins, we have not yet found an example of overlapping epitopes. Our approach differed from that of these other groups in that we have not used the peptides recognized by murine T cells to test for a corresponding response in humans. In addition, our results suggest that the specificity of the T-cell response may be influenced by the serotype of dengue virus eliciting the response, and we have studied a limited number of subjects, both human and murine, to date. Further studies will be necessary to determine if murine and human T cells can recognize the same dengue virus epitope(s).

These results indicate that the murine T-cell response to dengue virus includes the features that are hypothesized to relate to a T-cell-mediated immunopathological response to secondary dengue virus infection. Although some investigators have observed increased capillary permeability during dengue virus infection of mice (6), most investigators have failed to develop a murine model for DHF/DSS. This might relate to the failure of dengue virus to replicate to detectable levels in blood or tissues in mice (1), which would reduce the stimulus for the T-cell response, or to a reduced sensitivity of mice to the effects of T-cell activation and cytokine production, as has

TABLE 8. Recognition of E peptides by $CD8^+$ CTL clone 2D13

P815 target cell (peptide [2.5 μM])	Sequence	% Specific lysis[a]
Dengue-2 virus infected		20
No peptide (uninfected)		0
E(131-139)	QPENLEYTI	0
E(165-173)	TPQSSITEA	0
E(216-224)	LPLPWLPGA	0
E(218-226)	LPWLPGADT	1
E(221-229)	LPGADTQGS	2
E(331-339)	SPCKIPFEI	56
E(383-391)	EPGQLKLNW	4

[a] Underlined values are significantly higher than those for controls.

TABLE 9. Summary of functional phenotypes of dengue virus-specific T-cell clones studied

Example(s)	Cytolytic activity	MHC restriction	Serotype specificity	Protein recognized	Epitope recognized
$CD4^+$ clones					
2A1	No	$I\text{-}A^d$	D2	—[a]	—
2A2	Yes	$I\text{-}E^d$	D2 >> D4	—	—
2A3	No	$I\text{-}E^d$	D2	NS1	—
2A4	No	$I\text{-}E^d$	D4 > D3 > D2 > D1	—	—
2A5	No	$I\text{-}E^d$	D2	—	—
$CD8^+$ clones					
2E-56	Yes	—	D2	Pre-M	—
2D13	Yes	L^d	D2	E	331-339
2E-60	Yes	—	D2	E	—
2E-67	Yes	—	D2, D4	NS1/NS2A	—
2D42, 2D63, 2D65	Yes	K^d	D2, D4	NS3	296-310
2D7, 2E-8	Yes	—	D2[b]	—	—

[a] —, not determined.
[b] Recognition of other dengue virus serotypes was not definitively determined because the appropriate recombinant vaccinia viruses were not available.

been observed for endotoxin in some mouse strains (38). We are studying the possibility that these dengue virus-specific T-cell clones might permit a further analysis of this phenomenon.

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