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Dengue Virus-Specific Cross-Reactive CD8$^+$ Human Cytotoxic T Lymphocytes

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Stimulation with live dengue virus of peripheral blood mononuclear cells from a dengue virus type 4-immune donor generated virus-specific, serotype-cross-reactive, CD8$^+$, class I-restricted cytotoxic T lymphocytes (CTL) capable of lysing dengue virus-infected cells and cells pulsed with dengue virus antigens of all four serotypes. These CTL lysed autologous fibroblasts infected with vaccinia virus-dengue virus recombinant viruses containing the E gene or several nonstructural dengue virus type 4 genes. These results demonstrate that both dengue virus structural and nonstructural proteins are targets for the cytotoxic T-cell-mediated immune response to dengue virus and suggest that serotype-cross-reactive CD8$^+$ CTL may be important mediators of viral clearance and of virus-induced immunopathology during secondary dengue virus infections.

The severe manifestations of dengue virus infections, hemorrhage and shock, are most often seen after secondary infection with a heterologous serotype of dengue virus, implying a central role for the immune response in the pathogenesis of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Serotype-cross-reactive antibodies at subneutralizing concentrations form complexes with virions which bind to monocytes via Fc receptors, resulting in greater numbers of infected monocytes and high virus titers in vitro (14, 15). This phenomenon, known as immune enhancement, probably contributes to the pathogenesis of DHF-DSS but may be only part of the overall process. Less is known about the T-cell responses to dengue virus and its role in the immunity to and pathogenesis of DHF-DSS.

We have shown that CD4$^+$ T cells from dengue virus-immune humans and mice proliferate in response to soluble dengue virus antigens (22, 27). They produce gamma interferon (IFN-γ) (22), which up regulates Fc receptor expression on monocytes (12) and further increases infection by immune enhancement (19). In addition, we have recently detected serotype-cross-reactive, CD4$^+$ CD8$^-$ cytotoxic T-lymphocyte (CTL) clones, which are class II restricted and secrete IFN-γ (22a).

It is also important to identify dengue virus-specific, CD8$^+$, class I-restricted CTL and to define their functions. Evidence from studies in virus-infected animals indicates a necessary role for CD8$^+$ virus-specific CTL in recovery from certain virus infections (6, 23) and for inducing immunopathology (9, 23).

Little is known about CD8$^+$ cells in dengue virus infections. They do not seem to proliferate in response to soluble dengue virus antigens (27), and dengue virus-specific, CD8$^+$ CTL have not yet been described. However, T-cell responses to soluble antigens are usually of the CD4$^+$ variety (7, 18, 20, 24), probably because of the class II-restricted nature of soluble antigen processing. CD8$^+$ CTL responses have been detected to other viruses after stimulation with live virus (2) or with virus-infected fibroblasts (16). This is presumably due to a requirement for intracellular antigen processing, which appears to be important for optimal antigen presentation in the context of class I molecules (17, 26, 33).

In this report, we describe the proliferation of peripheral blood mononuclear cells (PBMC) from a dengue virus type 4-immune donor in response to live dengue virus and the generation of serotype cross-reactive, CD8$^+$, class I-restricted, dengue virus-specific CTL. Using vaccinia virus-dengue virus recombinant viruses, we show that the E protein and one or more of the nonstructural proteins are targets for lysis by these CTL. The results suggest that these CD8$^+$ serotype cross-reactive CTL may mediate viral clearance and contribute to shock by lysing dengue virus-infected cells in secondary infections.

MATERIALS AND METHODS

Human PBMC. Blood was obtained from a healthy donor who had been immunized with an experimental live dengue virus type 4 vaccine 1 year earlier. The human leukocyte antigen types of the lymphocytes are A2, 9; B35, 12; Cw4; DR7, w53; and DQw2. PBMC were separated by using Ficoll-Hypaque density gradient centrifugation and were frozen until use.

Viruses. Dengue virus type 1 (Hawaii strain) and type 2 (New Guinea C strain) were provided by Walter E. Brandt of the Walter Reed Army Institute of Research. Dengue virus type 3 (CH53489 strain) was supplied by Bruce I. Innis of the Armed Forces Research Institute of Medical Science, Bangkok, Thailand, and dengue virus type 4 (814669 strain) was obtained from Jack McCown of the Walter Reed Army Institute of Research. These viruses were propagated in C6/36 mosquito cells to titers of 10^7 to 10^8 PFU/ml as previously described (21) and stored at −70°C until use.

Dengue virus-vaccinia virus recombinants were obtained by recombining appropriate cDNA sequences of dengue virus type 4 strain 814669 with a pSC11 vaccinia virus intermediate vector and transfecting vaccinia virus strain WR-infected CV-1 cells with this DNA (4, 11, 35). The resulting recombinant viruses were isolated and plaque purified twice. Recombinant viruses were propagated in

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CV-1 cells, and titers ranged from 1×10^8 to 5×10^8 PFU/ml. Four recombinants were used in this study: A, which contains the genes for dengue virus type 4 NS1, NS2a, NS2b, NS3, NS4a, and 84% of NS4b; B, which includes the genes for dengue virus type 4 C, pre-M, E, NS1, and NS2a (35); C, which contains genes for dengue virus type 4 NS1 and NS2a (11); D, which is vaccinia virus containing the *lacZ* gene (8) and which was used as a control; and E, which contains the gene for dengue virus type 4 E protein (4).

Preparation of dengue virus antigens. Dengue virus antigens were prepared as described previously (22). Vero cells grown in plastic flasks (Becton Dickinson Labware, Oxnard, Calif.) were inoculated with dengue virus at a multiplicity of infection of 1 and cultured in minimal essential medium containing 2% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) until 50% of the cell monolayer exhibited cytopathic effects. Cells were then scraped from the flasks with rubber cell scrapers (Costar, Cambridge, Mass.), washed three times with phosphate-buffered saline at 4°C, treated with 0.025% glutaraldehyde (Sigma, St. Louis, Mo.) in phosphate-buffered saline at 4°C for 15 min, washed three more times with phosphate-buffered saline, and suspended in RPMI 1640. Cells were then sonicated with a sonic dismembrator (Fisher Scientific Co., Pittsburgh, Pa.) for 2 to 3 min and were finally centrifuged at $1,600 \times g$ for 10 min. The supernatants were collected and stored at −70°C until used. Control antigens were prepared the same way by using uninfected Vero cells. Fifteen 75-cm^2 flasks were used to prepare 3-ml samples of antigens.

Stimulation of PBMC. A total of 4×10^6 PBMC from a dengue virus type 4-immune donor were suspended in 1 ml of RPMI 1640 containing penicillin-streptomycin, glutamine, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma), and 10% heat-inactivated pooled human AB serum (Hazelton Research Products, Inc., Lenexa, Kans.) and placed into wells of 24-well cluster plates (Costar). One milliliter of dengue virus containing 10^7 to 10^8 PFU was added to each well, and the plates were incubated for 6 to 9 days at 37°C with 5% CO_2 in a humid incubator. Mock stimulation was carried out by using uninfected mosquito cell supernatants.

Cell lines. Skin fibroblast cultures were established by using a 3-mm biopsy sample of skin which was minced. Cells were passed several times in 75-cm^2 flasks before samples were frozen and later thawed and used as targets. These cells were maintained in minimal essential medium with 10% fetal calf serum with the same additives as listed above.

Lymphoblastoid cell lines (LCL) were established by infecting PBMC with Epstein-Barr virus obtained from an infected marmoset cell line (29) provided by Takeshi Sairenji of University of Massachusetts Medical Center. LCL were maintained in RPMI containing 10% fetal calf serum and the same additives as listed above.

Infection and antigen pulsation of target cells. A total of 1×10^5 to 2×10^5 fibroblasts were suspended in 100 μl of minimal essential medium in 15-ml conical centrifuge tubes and mixed with 100 μl of virus or uninfected mosquito cell supernatant and incubated for 1.5 to 2 h at 37°C with occasional agitation. The cells were then diluted, and 2 ml was seeded into wells of 24-well cluster plates overnight for use as target cells the next morning. The multiplicities of infection used were between 10 and 50 for dengue virus and between 2 and 5 for the dengue virus-vaccinia virus recombinants.

Antigen pulsation was performed after seeding 10^5 LCL or fibroblasts in 24-well cluster plates in a volume of 0.5 ml. Soluble dengue virus antigens were added at final dilutions between 1:20 and 1:200 15 to 18 h before cytotoxicity assays. In some experiments, beta interferon (Lee Biomolecular, San Diego, Calif.) was added at a final concentration of 5,000 U/ml 2 to 3 days before antigen pulsation. This has been shown to render target cells resistant to lysis by NK cells (31) and to increase their sensitivity to CTL (5).

Cell surface antigen analysis. Anti-Leu2 (CD8) and anti-Leu3 (CD4) antibodies directly conjugated to fluorescein isothiocyanate (Becton Dickinson Co., Mountain View, Calif.) were used to quantitate $CD8^+$ and $CD4^+$ PBMC. Briefly, 20 μl of antibody was mixed with 10^5 to 10^6 cells in a volume of 50 μl and incubated for 45 to 60 min at 4°C. Cells were then washed twice in cold RPMI 1640 and were then analyzed on a fluorescence-activated cell sorter (FACS 440; Becton Dickinson Co.) or by fluorescence microscopy.

Antibody-complement depletion. Anti-OKT4 (CD4) and anti-OKT8 (CD8) antibodies (Ortho Diagnostic Systems, Inc., Raritan, N.J.) and anti-Leu 11b (CD16; Becton Dickinson Co.) were used in antibody-complement depletion experiments. A total of 1×10^6 to 2×10^6 virus-stimulated PBMC were suspended in 50 μl, and then 100 μl of antibody diluted 1:15 was added, followed by incubation at 4°C for 30 min. The cells were washed twice in cold RPMI 1640 and suspended in 100 μl, to which 200 μl of rabbit complement diluted 1:3 (Cedarlane Laboratories, Ontario, Canada) was added, followed by incubation for 1 h at 37°C. Cells were then washed three times in RPMI 1640 and were used in cytotoxicity assays.

Cytotoxicity assays. A total of 10^5 to 10^6 target cells were labeled with 0.5 mCi of ^{51}Cr (Na_2CrO_4) (Dupont, NEN Research Products, Boston, Mass.) in 0.2 ml of RPMI 1640 containing 10% fetal calf serum at 37°C for 1 h. The cells were then washed three times and suspended at 10^4 cells per ml for fibroblasts and 2.5×10^4 cells per ml for LCL and added to 96-well round-bottomed microdilution plates (Costar) in a volume of 100 μl in triplicate. Effector cells were added in a volume of 100 μl for a final volume of 200 μl. After incubation for 4 to 6 h, the plates were centrifuged at $200 \times g$ for 5 min, and supernatant fluids were collected and counted in a gamma counter (Packard, Sterling, Calif.). The percent specific release was calculated by the formula [(cpm experimental release) − (cpm spontaneous release)]/[(cpm maximum release) − (cpm spontaneous release)] × 100, in which cpm is counts per minute. The spontaneous release of label was between 11 and 30%. Maximum release was assessed by treatment of labeled cells with detergent.

Antibody blocking of cytotoxicity. W6/32 (Accurate Chemical and Scientific Co., Westbury, N.Y.) and OKIa1 (Ortho) are monoclonal antibodies which recognize framework determinants of human leukocyte antigen A, B, and C, and DR determinants, respectively. A 50-μl portion of antibody diluted 1:20 was added to 50 μl of ^{51}Cr-labeled target cells (2×10^3 to 5×10^3/ml) in 96-well round-bottomed plates for 10 min, followed by addition of 100 μl of effector cells. The assays were 4 h long.

Proliferation assay. A 100-μl sample of cells from 6-day-old PBMC bulk cultures was added to 96-well microdilution wells in triplicate and pulsed with 1.25 μCi of tritiated thymidine for 4 to 16 h before harvest. Cells were harvested by using a Titertek Multiharvester (Skatron, Inc., Sterling, Va.), and [^{3}H]thymidine incorporation was counted in a liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.).

TABLE 1. Stimulation of dengue virus type 4-immune PBMC with live and UV-inactivated dengue virus

Expt. no.	Stimulus	[^{3}H]thymidine (cpm)	Stimulation index
1	Dengue virus type 4	26,712	5.6
	Dengue virus type 2	13,137	2.7
	Mock[a]	4,789	
2	Dengue virus type 4	19,652	16.0
	Dengue virus type 4[b]	627	0.5
	Mock[a]	1,209	

[a] Uninfected mosquito cell supernatants were used for mock stimulation.
[b] UV inactivated.

TABLE 3. Characterization of cytotoxic activity[a]

Expt no.	Dengue virus type 4-stimulated PBMC treatment	% Specific ^{51}Cr release from dengue virus type 2-infected fibroblasts	% Inhibition
1	Medium	65	
	W6/32	34	48
	OKIa-1	55	15
2	Complement	46	
	anti-CD8 + complement	18	61
	anti-CD4 + complement	50	−9
	anti-Leu 11b + complement	44	4

[a] Effector-to-target ratio was 100:1 in a 4-h assay.

RESULTS

Stimulation of dengue virus type 4-immune PBMC with live or UV-inactivated dengue virus. PBMC taken from a dengue virus type 4-immune donor were placed in culture with either live dengue virus or virus which had been exposed to UV light and shown to have no plaque-forming capacity. After 6 days, the cells were pulsed with [^{3}H]thymidine, and proliferation was assessed. The results in Table 1 show that the dengue virus type 4-immune PBMC responded best to dengue virus type 4. There was also a lower but significant response to dengue virus type 2, which indicated that the response was serotype cross-reactive. Nonimmune PBMC did not respond (data not shown), and UV-inactivated dengue virus type 4 failed to generate a response.

Lysis of dengue virus-infected fibroblasts by dengue virus type 4-immune PBMC stimulated by live dengue virus. PBMC from a dengue virus type 4-immune donor were tested for cytotoxic activity against dengue virus-infected autologous fibroblasts after 7 to 9 days of stimulation with dengue virus. Fibroblasts were chosen as target cells to detect CD8$^+$ CTL because they do not constitutively express class II major histocompatibility complex antigens (25). Dengue virus type 4-immune PBMC stimulated with either dengue virus type 2 or dengue virus type 4 lysed fibroblasts infected with either serotype (Table 2). Uninfected cells were also lysed, but the levels of lysis were always lower than those against virus-infected targets. These results show that the cytotoxic response is serotype cross-reactive.

Characterization of cytotoxic activity. Antibody-blocking and antibody-complement depletion studies were carried out to determine the identity of the killer cells. Inclusion in the cytotoxicity assay of an antibody against class I antigens, W6/32, inhibited the killing of dengue virus type 2-infected fibroblasts by 48% (Table 3), which indicates that much of the killing was class I antigen restricted. An antibody to class II DR antigens, OKIa-1, inhibited the killing poorly, showing that the killing was not DR restricted. Since fibroblasts do not express any class II antigens under these conditions (25), killing restricted by the other class II antigens (DP and DQ) can also be excluded.

Treatment of the effector population with anti-CD8 and complement resulted in the death of 37% of these cells and reduced their lytic capacity against dengue virus type 2-infected fibroblasts by 61% (Table 3). Anti-CD4 and complement treatment similarly killed 35% of the effector population but failed to inhibit the cytotoxic activity (Table 3). Anti-Leu 11b and complement treatment failed to induce either a significant decrease in viability (data not shown) or a reduction in the cytotoxic capacity of effector cells (Table 3). Taken together, these results indicate that the effector cells in this system were virus-specific, CD8$^+$, HLA class I-restricted, serotype-cross-reactive CTL.

Cross-reactive lysis of dengue virus antigen-pulsed LCL and fibroblasts. It is difficult to infect an adequate percentage of human LCL and fibroblasts in vitro with dengue virus types 1 and 3; therefore, we pulsed fibroblasts and LCL with dengue virus antigens prepared from dengue virus-infected, glutaraldehyde-fixed Vero cells and used them as targets for dengue virus-stimulated PBMC.

Fibroblasts and LCL pulsed with dengue virus antigens were efficiently lysed by dengue virus type 4-stimulated PBMC, but lysis of control antigen-treated or untreated fibroblasts was minimal (Table 4). Thus, these CTL are cross-reactive against all four dengue virus serotypes.

We characterized the major histocompatibility complex restriction of killing against LCL by including antibodies to class I (W6/32) and class II (OKIa-1) antigens in the cyto-

TABLE 2. Lysis of dengue virus-infected fibroblasts by dengue virus type 4-immune PBMC stimulated with dengue virus

Expt. no	PBMC stimulus	% Specific ^{51}Cr release from fibroblasts infected with:		
		Dengue virus type 4	Dengue virus type 2	No virus (mock)[a]
1[b]	Dengue virus type 4	26	26	2
	Dengue virus type 2	28	24	7
	Mock[a]	2	5	2
2[c]	Dengue virus type 4	62	58	2

[a] Uninfected mosquito cell supernatant.
[b] Effector-to-target ratio was 40:1 in a 4-h assay.
[c] Effector-to-target ratio was 100:1 in a 4-h assay.

TABLE 4. Lysis of dengue virus antigen-pulsed LCL and fibroblasts[a]

Target cell type	% Specific release of target cells treated with antigens from dengue virus type:				
	1	2	3	4	Control[b]
Fibroblasts	21	56	27	19	5
Fibroblasts	ND[c]	ND	ND	36	5
LCL	ND	ND	ND	52	7

[a] Effectors were dengue virus type 4-immune PBMC stimulated for 8 days with dengue virus type 4. The effector-to-target ratio was 100:1, and the assay length was 6 h. Target cells pulsed with dengue virus antigens or control antigens were used as targets the following day.
[b] Control antigen, Uninfected mosquito cell supernatant.
[c] ND, Not done.

TABLE 5. Lysis of fibroblasts infected with dengue virus-vaccinia virus recombinants[a]

Infecting virus or recombinant (proteins)	% Specific ^{51}Cr release in:	
	Expt 1	Expt 2
Dengue virus type 2	94	ND[b]
Dengue virus type 4	72	31
A (NS1, NS2a, NS2b, NS3, NS4a, NS4b)	39	17
B (C, pre-M, E, NS2, NS2a)	8	13
C (NS1, NS2a)	−2	0
Vaccinia virus control[c]		
E (E)	13	28
None (mock infection)	−4	−1

[a] Effectors were from dengue virus type 4-immune PBMC stimulated for 8 days with dengue virus type 4. Assay length was 4 h. Effector-to-target ratio was 100:1 in experiment 1 and 40:1 in experiment 2.

[b] ND, Not done.

[c] Lysis of targets infected with vaccinia virus was 12% in both experiments, which was subtracted for clarity.

toxicity assays. W6/32 inhibited the killing by 57%, whereas OKIa-1 had no effect. These data and the fact that fibroblasts, which lack class II antigens, are killed argue strongly that this lysis is mediated by class I-restricted CTL.

Lysis of fibroblasts infected with dengue virus-vaccinia virus recombinants. We examined the proteins which are targets for dengue virus-specific class I-restricted CTL by infecting fibroblasts with dengue virus-vaccinia virus recombinant viruses, each of which contained a different portion of the dengue virus type 4 genome. Recombinant A contained genes which code for dengue virus proteins NS1, NS2a, NS2b, NS3, NS4a, and 84% of NS4b. Recombinant B contained genes coding for C, pre-M, E, NS1, and NS2a. Recombinant C included genes coding for NS1 and NS2a, and recombinant E contained genes coding for the E protein. Recombinant D is the parental vaccinia virus which contains the *lacZ* gene and has none of the dengue virus genome and served as a control. CTL lysed fibroblasts infected with recombinants A, B, and E, but not those infected with C and D (Table 5). These results show that E is a target protein for these CTL but that NS1 and NS2a are not. The data also imply that at least one of the NS2b, NS3, NS4a, or NS4b proteins is a target for these CTL. From these studies, we cannot determine whether C, pre-M, and NS5 contain target epitopes for CTL. Recombinant vaccinia viruses which contained the genes for the dengue virus type 2C, pre-M, and E proteins and the genes for dengue virus type 2 NS1 and NS2a proteins were also used to infect autologous fibroblasts. Consistent with the concept that these CTL are cross-reactive, fibroblasts infected with the dengue virus type 2 recombinant expressing C, pre-M, and E proteins were lysed by dengue virus type 4-stimulated, dengue virus type 4-immune PBMC. However, these PBMC did not lyse fibroblasts infected with the dengue virus type 2 recombinant expressing NS1 and NS2a (data not shown).

DISCUSSION

In this report, we describe serotype-cross-reactive, $CD8^+$ $CD4^-$, class I-restricted, dengue virus-specific CTL obtained after stimulation with live virus of PBMC from a dengue virus-immune individual. The evidence that lysis was mediated by class I-restricted $CD8^+$ CTL is as follows: (i) antibody and complement treatment of the effectors using anti-CD8 or anti-CD3 but not anti-CD16 or anti-CD4 depleted the killing (Table 3); (ii) antibody to class I but not class II antigens decreased the level of lysis (Table 3); (iii) virus-infected fibroblasts, which do not express major histocompatibility complex class II antigens (25), were specifically lysed (Tables 2, 4, and 5). In other viral systems (2, 16), the use of live virus or virus-infected fibroblasts has also been necessary to induce class I-restricted $CD8^+$ CTL. This may be due to the requirement for intracellular antigen synthesis sometimes required for optimal presentation of antigen in the context of class I molecules (17, 26, 33). Stimulation of PBMC with soluble dengue virus antigens generated $CD4^+$ class II-restricted CTL (22a). We failed to induce $CD8^+$ CTL with soluble dengue virus antigens despite inducing good levels of proliferation.

However, fibroblasts and LCL pulsed with these antigens were sensitive to class I-restricted CTL-mediated lysis. This appears to be consistent with a report which showed that influenza virus proteins could bind to class I antigens displayed on the plasma membrane and that these antigen-pulsed targets were sensitive to lysis by influenza virus-specific, $CD8^+$, class I-restricted CTL (32).

A rather large amount of dengue virus was necessary for optimal CTL induction (10^7 to 10^8 PFU). During secondary dengue virus infections, the increased amount of virus present due to antibody-mediated enhancement of infection in Fc receptor-positive cells and the presence of memory CTL may result in a more vigorous CTL response than is usually present during primary infections. This CTL response may contribute both to the enhanced destruction of virus-infected cells and to immunopathology. Although DHF-DSS are more commonly observed during secondary dengue virus infections, some infants 6 to 12 months of age with maternally derived enhancing antibodies develop DHF-DSS during primary dengue virus infections (13). This may be triggered by a primary CTL response against an augmented number of infected monocytes.

In order to postulate that CTL play a role in viral clearance and DHF-DSS during secondary dengue virus infections, they must be serotype cross-reactive with regard to specificity for induction and lysis. Stimulation by dengue virus type 2 of PBMC from a dengue virus type 4-immune individual induced proliferation of CTL capable of lysing dengue virus type 2-infected targets (Tables 1 and 2). Stimulation of these PBMC with dengue virus type 4 resulted in CTL capable of lysing target cells expressing all four serotypes of dengue virus (Table 4), thus demonstrating the cross-reactive nature of these CTL.

Our bulk culture studies revealed the presence of cross-reactive CTL. We have recently isolated 12 $CD8^+$ $CD4^-$ $CD16^-$ CTL clones from a dengue virus type 4-immune individual which were capable of lysing dengue virus type 2-infected targets (unpublished data), demonstrating cross-reactivity at the clonal level. We have also described clones of serotype-cross-reactive, class II-restricted, $CD4^+$ $CD8^-$ CTL, using soluble dengue virus antigens as a stimulus (22a). These clones proliferated and produced IFN-γ in response to soluble antigens from all four serotypes. It will be useful to compare class I- and class II-restricted clones with regard to fine specificity and lymphokine production in order to better understand their roles in recovery from dengue virus infection and in the pathogenesis of DHF-DSS.

DHF-DSS is usually observed in individuals undergoing a secondary infection with a different serotype of dengue virus than that which caused the primary infection. Thus, it is likely that cross-reactive immune responses are responsible for the pathogenesis of DHF-DSS. T cells may be involved

in several ways. Production of IFN-γ (22, 22a) by $CD4^+$ CTL leads to an increase in Fc receptor expression on monocytes (12), which results in a greater number of infected monocytes by virus-antibody complexes (19). These monocytes may then be susceptible to lysis by class I- and class II-restricted CTL, resulting in the release of vasoactive factors such as complement components (3) and tumor necrosis factor (10). T cells may also play a role in DHF-DSS by producing interleukin 2. Interleukin 2 augments NK cell activity and proliferation (30). NK cells can mediate ADCC (21) and produce IFN-γ as well as tumor necrosis factor (10, 30), which may further contribute to DHF-DSS (1).

Currently there is no effective vaccine against dengue virus. In order to design a safe and effective vaccine and to understand the pathogenesis of DSS, it is important to learn which dengue virus-coded proteins induce protective T-cell responses and which responses may lead to DHF-DSS. Our studies using dengue virus type 4-vaccinia virus constructs indicate that the E protein and one or more of the NS2b, NS3, NS4a, and NS4b proteins are targets for $CD8^+$ CTL. Since the E protein also contains epitopes for cross-reactive antibodies which can mediate immune enhancement (14), there is concern about using it in a vaccine. NS proteins are not expressed in virions and may be candidates for use in a subunit vaccine. It is therefore important to elucidate which NS proteins are recognized by cross-reactive T cells and which NS proteins are targets for CTL-mediated clearance and/or CTL-mediated DHF-DSS.

NS1 or NS2a do not seem to contain CTL epitopes in this system, despite the facts that NS1 is a highly conserved protein among all four dengue virus serotypes and that it induces a serotype cross-reactive antibody response (28). Immunization of mice with NS1 protein protects mice against intracerebral challenge with dengue virus, but the protection is not serotype cross-reactive (28). Recently, one of us reported that immunization with a cell lysate containing dengue virus proteins C, pre-M, E, NS-1, and NS2a protected mice against dengue virus encephalitis (34). Mice immunized with this preparation develop antibody responses to NS1 but lack consistently detectable virus neutralizing antibodies. Mice immunized with a dengue virus-vaccinia virus recombinant containing the gene for dengue virus type 4 E protein also resisted a fatal challenge with dengue virus type 4; anti-dengue virus antibody titers were either low or undetectable in these mice (4). Cell-mediated immune responses were not analyzed in those experiments, and the mechanism of protection remains speculative.

Further studies are needed to better define the proteins and epitopes which are recognized by dengue virus-specific CTL and their major histocompatibility complex haplotype restrictions. This information could be important in determining which elements of the CTL response contribute to recovery from dengue virus infection and to the severe complications of dengue virus infections.

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