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Recognition of Envelope Protein by Dengue Virus Serotype-Specific Human CD4$^+$ CD8$^-$ Cytotoxic T-Cell Clones

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We analyzed dengue virus-specific CD4$^+$ CD8$^-$ cytotoxic T lymphocytes (CTL) at the clonal level to further understand their role in dengue virus infections. Stimulation of peripheral blood mononuclear cells from two dengue virus type 4 (D4V)-immune donors with live D4V or noninfectious D4V antigen generated 17 HLA class II-restricted CD4$^+$ CTL capable of specific lysis of dengue virus antigen-treated autologous lymphoblastoid cell lines. Thirteen clones were D4V specific, three clones were cross-reactive for D2V and D4V, and one clone was cross-reactive for D1V, D3V, and D4V. Antigen recognition by six D4V-specific clones and three D2V- and D4V-cross-reactive clones was restricted by HLA-DR7. Five D4V-specific CD4$^+$ CTL clones lysed autologous lymphoblastoid cell lines infected with a dengue virus-vaccinia virus recombinant containing the E gene of D4V, whereas three serotype-cross-reactive CTL clones did not. These results indicate that E-specific clones are serotype specific and HLA-DR7 restricted in these two donors and suggest that a common epitope on E protein may be recognized. E protein-specific CD4$^+$ CTL may be important mediators of virus clearance especially during reinfection with the same serotype as that in primary infection by providing help for virus-specific antibody production and lysis of virus-infected cells.

Dengue is a viral illness that causes extensive morbidity throughout epidemic tropical areas of the world and is a significant cause of mortality in Southeast Asia (9). Dengue viruses are mosquito-borne flaviviruses which have been subgrouped into four antigenically related serotypes, dengue virus types 1, 2, 3, and 4 (D1V, D2V, D3V, and D4V, respectively) (14). Dengue virus infections may be asymptomatic, cause a self-limited febrile illness known as dengue fever, or, in a small percentage of cases, cause a severe illness known as dengue hemorrhagic fever (DHF) (8). Epidemiological studies have shown that the incidence of DHF is much greater in individuals experiencing secondary infections with a serotype of dengue virus distinct from the virus serotype that caused the primary infection (8).

Primary infection with a dengue virus appears to confer lifelong protection against reinfection with a dengue virus of the same serotype (32). Presumably, this protection is generated through production of virus-specific neutralizing antibodies and also possibly by serotype-specific memory CD4$^+$ and CD8$^+$ T cells. When secondary infections with homotypic virus occur in the absence of sufficient levels of specific neutralizing antibodies, the infecting virus will multiply at the site of entry, anamnestic immune responses will be induced, and virus will be cleared. However, during secondary infection with a serotype different from that which caused primary infection, there is increased risk for more severe disease (8, 10).

Virus-specific antibody responses require help from CD4$^+$ T lymphocytes. The presence of an early serotype-cross-reactive immunoglobulin G (IgG) response to secondary dengue virus infection predicts the existence of serotype-cross-reactive memory CD4$^+$ T lymphocytes (8, 11). We have previously reported the characterization of dengue virus-specific, serotype-cross-reactive CD4$^+$ and CD8$^+$ human T lymphocytes which predominantly recognize nonstructural proteins (18, 22, 24). To date, structural protein-specific, serotype cross-reactive human CD4$^+$ T lymphocytes have not been reported.

Serotype-specific CD4$^+$ T lymphocytes may play an important role in protective immunity against reinfection with the same serotype of dengue virus. In this paper, we describe the isolation and characterization of D4V-specific CD4$^+$ cytotoxic T-lymphocyte (CTL) clones generated from two donors. Most of these D4V-specific clones recognize envelope (E) protein, while none of the serotype-cross-reactive clones recognize E protein. E protein contains epitopes recognized by serotype-specific neutralizing antibodies (7, 13). The presence of serotype-specific CD4$^+$ T-cell epitope(s) on E protein suggests that T-cell immune responses to E protein may contribute to protection against reinfection with the homologous serotype of dengue viruses.

MATERIALS AND METHODS

Flaviviruses. D1V (Hawaii strain), D2V (New Guinea C strain), D3V (CH53489 strain), D4V (814669 strain), yellow fever virus (17D strain), and West Nile virus (E101 strain) were used in this study.

These viruses were propagated in C6/36 mosquito cells as previously described (20). Briefly, C6/36 cell monolayers were infected at a multiplicity of infection of 0.1 PFU per cell and were incubated for 7 days at 28°C in minimal essential medium containing 2% fetal calf serum (FCS) and 0.8% bovine serum albumin, and the supernatant was collected and stored at −70°C. Viral titers of supernatants were estimated to be 10^7 to 10^8 PFU/ml by plaque assays on Vero cell monolayers.

Preparation of noninfectious flavivirus antigens. D1V, D2V, D3V, D4V, yellow fever virus, and West Nile virus antigens were prepared from infected Vero cell monolayers as

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previously described (22). Briefly, Vero cell monolayers were infected at a multiplicity of infection of 1 PFU per cell and were incubated at 37°C in minimal essential medium supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–2 mM glutamine–100 U of penicillin per ml–100 μg of streptomycin per ml containing 2% FCS until 50% of the cells displayed cytopathic effects. Cells were then harvested by scraping, washed, fixed with 0.025% glutaraldehyde in phosphate-buffered saline (PBS) for 15 min on ice, washed again, and resuspended at 3×10^8 cells per ml in RPMI. The suspension of fixed cells was then sonicated on ice with a sonic dismembrator (Fisher Chemical Co., Pittsburgh, Pa.) for 3 min and was centrifuged at $1,600 \times g$ for 10 min at 4°C. The supernatant was collected, aliquoted, and frozen at −70°C as viral antigen. Control antigen from uninfected Vero cell monolayers was prepared similarly.

DV-VV. Recombinant dengue-vaccinia viruses (DV-VV) were constructed by insertion of dengue viral genome segments from D4V (814669 strain) into the pSC11 vaccinia virus vector under control of the vaccinia virus P7.5 early-late promoter as previously described (2, 31, 34).

PBMC. Human peripheral blood mononuclear cells (PBMC) were obtained from two healthy donors, donors 1 and 2, who had been immunized with the D4V strain 814669 as a candidate live vaccine 1 year earlier (15). The sera of donors 1 and 2 did not contain antibody to dengue viruses before immunization. Donor 1 did not have any significant clinical symptoms; however, viremia was detected on day 9, and donor 1 developed D4V-specific neutralizing antibodies with a titer of 1:90 on day 30 after immunization. Donor 2 did not develop any significant clinical symptoms; however, viremia was detected on days 12 to 15, and donor 2 developed D4V-specific neutralizing antibodies with a titer of 1:30 on day 30 after immunization. PBMC were collected by leukopheresis 1 year after immunization and were isolated by Ficoll-Hypaque density gradient centrifugation. These PBMC were then resuspended in RPMI containing 20% FCS and 10% dimethyl sulfoxide and were frozen in a programmable cryopreservation chamber (CRYO-MED, Mt. Clemens, Mich.). The HLA alleles of donor 1 were A2, A23, B35, Cw4, DR7, DRw53, and DQw2, and those of donor 2 were A2, A28, B51, Bw57, Cw6, DR5, DR7, DRw52, DRw53, DQw1, and DQw2.

Establishment of dengue virus-specific T-cell clones. PBMC from donor 1 were suspended at 4×10^6 cells per ml in RPMI containing 20% heat-inactivated human AB serum (HABS; Hazelton Research Products, Inc., Lenexa, Kans.). A 1.0-ml volume of cell suspension was added to 1.0 ml of D4V in 24-well cluster plates (Costar, Cambridge, Mass.), as previously described (3). Dengue virus-specific, class II-restricted CTL clones were propagated from limiting dilutions of bulk cultures on day 9 at 10 to 100 cells per well. Limiting-dilution cultures were set up in 96-well round-bottom plates (Costar) with each well containing 100 μl of AIM-V medium containing 20% HABS (Advanced Biotechnologies, Inc., Columbia, Md.), 20% TCGF, and 10^5 gamma-irradiated autologous PBMC and 100 μl of D4V. Wells demonstrating sufficient growth and cell density at each 7-day period were restimulated in 48-well plates with 0.5 ml of AIM-V medium containing 20% HABS, 20% TCGF, and 10^6 gamma-irradiated autologous PBMC and 0.5 ml of D4V. On day 3 postrestimulation, 48-well plates were fed with 0.5 ml of AIM-V containing 20% HABS, 20% TCGF, and 10 U of recombinant human interleukin-2. Limiting-dilution culture of 192 seeded wells produced 22 clones which grew in 48-well plates. A total of 21 clones were determined to be $CD3^+$ $CD4^+$ $CD8^-$ by fluorescence microscopy analyses. $CD4^+$ CTL clones were similarly established from PBMC of donor 2 by using noninfectious D4V antigen instead of live D4V as previously described (24). Two clones were established from a 96-well limiting-dilution culture at 1 cell per well.

Phenotypic analysis. CTL clones were washed twice in cold PBS. Fluorescein isothiocyanate-conjugated antibodies (Lee Biomolecular, San Diego, Calif.) were added to 10^5 cells at a final dilution of 1:20. Conjugated antibodies used were anti-Leu2 (anti-CD8), anti-Leu3 (anti-CD4), and anti-Leu4 (anti-CD3). Control cells were labeled with conjugated normal mouse IgG. Labeled CTL were then washed three times in ice-cold PBS, and surface expression of CD3, CD4, and CD8 was qualitatively analyzed by fluorescence microscopy.

Preparation of target cells. Autologous B lymphoblastoid cell lines (LCL) were derived through transformation of peripheral blood lymphocytes with Epstein-Barr virus as previously described (18) and were maintained in RPMI containing 10% FCS at 37°C. Culture fluid of Epstein-Barr virus-transformed marmoset cell line B95-8 was used as a source of Epstein-Barr virus.

Antigen-treated LCL target cells were prepared by seeding 2×10^5 cells per well in 24-well cluster plates in a final volume of 0.5 ml of RPMI containing 10% FCS and dengue virus antigen diluted 1:50 to 1:75. Cells were incubated for 16 h with antigen. DV-VV-infected target cells were prepared by infecting 10^6 LCL with DV-VV at a multiplicity of infection of approximately 20 PFU per cell for 2 h. After being washed twice, LCL were cultured in RPMI containing 10% FCS at 37°C for 16 to 20 h and were used as target cells.

Target cells were washed twice in RPMI containing 10% FCS and were labeled by incubation with 0.25 mCi $Na_2\,{}^{51}CrO_4$ (New England Nuclear Research Products, Boston, Mass.) for 45 to 60 min in 100 μl at 37°C. After four washes to remove excess ^{51}Cr, target cells were counted and resuspended at 10^4 cells per ml for use in cytotoxicity assays.

Cytotoxicity assays. Cytotoxicity assays were performed in 96-well round-bottom plates to which 10^3 labeled targets were added in 100 μl of RPMI containing 10% FCS. A 100-μl volume of effector cells in RPMI containing 10% FCS was subsequently added. All assays were performed in triplicate or quadruplicate. Following centrifugation at $200 \times g$ for 5 min, the plates were incubated at 37°C for 5 h. Supernatant fluids were harvested, and ^{51}Cr content was measured in a gamma counter (Packard, Sterling, Calif.). The percent specific release was calculated as (cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release) × 100, where cpm is counts per minute.

RESULTS

Establishment of D4V-specific $CD4^+$ CTL clones. Fifteen $CD4^+$ $CD8^-$ T-cell clones and two $CD4^+$ $CD8^-$ CTL clones were established from PBMC of the D4V-immune donors 1 and 2, respectively, as described in Materials and Methods. Twelve of the 15 $CD4^+$ CTL clones derived from donor 1 were specific for D4V, and 3 clones were cross-reactive for D2V and D4V. One of the two $CD4^+$ CTL clones derived from PBMC of donor 2 was specific for D4V, and one clone was cross-reactive for D1V, D3V, and D4V. Table 1 presents data on the D4V-specific and serotype-cross-reactive lysis of target cells by 10 representative $CD4^+$ $CD8^-$ CTL clones. None of these clones recognized yellow fever virus or West Nile virus, which are related flaviviruses (data not presented).

HLA-DR7-restricted recognition of D4V by $CD4^+$ CTL clones. The HLA class II alleles of donor 1 and donor 2 were DR7, DQw2, and DRw53 and DR5, DR7, DQw2, DQw3, DRw52, and DRw53, respectively. The HLA restriction of nine

TABLE 1. Dengue virus serotype specificities of $CD4^+$ $CD8^-$ T-cell clones established from donors 1 and 2[a]

Clone[b]	% Specific ^{51}Cr release[c]				
	D1V	D2V	D3V	D4V	Control antigen
Serotype specific					
CB6.1	24	10	12	67	16
CB6.3	1	0	0	70	0
CB6.15	0	2	0	75	3
CB6.21	0	10	0	54	0
CB6.22	2	5	0	80	1
CP5	0	0	0	65	0
Serotype cross-reactive					
CB6.18	7	51	0	16	5
CB6.19	1	38	0	22	1
CB6.20	9	62	0	22	0
CP3	31	0	44	33	0

[a] A total of 10^3 target cells were incubated with effector cells for 5 h at an effector/target ratio of 10:1.
[b] Clones CB6.1, CB6.3, CB6.15, CB6.21, CB6.22, CB6.18, CB6.19, and CB6.20 were established from the PBMC of donor 1, and clones CP3 and CP5 were established from the PBMC of donor 2.
[c] The percentage of specific ^{51}Cr release was calculated by the formula described in Materials and Methods.

dengue virus-specific CTL clones was determined in cytotoxicity assays with HLA class II-defined allogeneic LCL as target cells. All of the $CD4^+$ CTL clones which originated from donor 1, including the five D4V-specific clones and three clones cross-reactive for D2V and D4V, lysed dengue antigen-treated allogeneic target cells which shared HLA-DR7 (Table 2). A clone CP5 derived from donor 2 also lysed dengue virus antigen-cultured allogeneic target cells which shared HLA-DR7 (Table 3). These results indicate that all of these nine $CD4^+$ CTL clones were HLA-DR7 restricted.

Recognition of E protein by D4V-specific $CD4^+$ CTL clones. The dengue virus protein specificities of these dengue virus-specific $CD4^+$ CTL clones were examined by using recombinant vaccinia viruses which contain genome coding for proteins of D4V or D2V. Five D4V-specific clones, CB6.1, CB6.3, CB6.15, CB6.21, and CP5, lysed target cells infected with recombinant vaccinia virus which expressed the E protein of D4V but did not lyse target cells infected with recombinant vaccinia viruses which expressed the NS3 protein of D4V; NS1 and NS2a of D4V; or C, pre-M, and E proteins of D2V (Table

TABLE 2. HLA-DR7-restricted recognition of D4V antigen by $CD4^+$ CTL clones established from donor 1[a]

Expt and target cell[b]	Shared HLA class II allele(s)	% Specific ^{51}Cr release[c]							
		D4V specific clones					D2V- and D4V cross-reactive clones		
		CB6.1	CB6.3	CB6.15	CB6.21	CB6.22	CB6.18	CB6.19	CB6.20
Expt 1									
Autologous	DR7, DQw2, DRw53	24	28	59	27	46	45	57	58
CP	DR7, DQw2, DRw53	15	22	30	12	37	21	39	48
9049	DR7, DQw2, DRw53	42	45	47	43	55	ND[d]	ND	ND
PG	DQw2	0	1	3	3	0	0	0	0
FE	DQw2	0	0	3	2	0	0	1	1
9038	None	0	0	0	ND	0	1	2	2
Expt 2									
Autologous	DR7, DQw2, DRw53	21	ND	31	15	35	27	28	38
9052	DR7, DRw53	28	ND	49	17	57	31	35	44
VAVY	DQw2	0	ND	0	0	0	0	1	2
Expt 3									
Autologous	DR7, DQw2, DRw53	59	62	68	72	75	32	40	51
9049	DR7, DQw2, DRw53	ND	ND	ND	ND	ND	25	30	34
9087	DQw2	ND	ND	ND	ND	ND	0	0	0
JC	DRw53	0	1	2	4	1	3	0	0
MS	DRw53	0	0	0	8	4	ND	ND	ND
9029	DRw53	ND	ND	ND	ND	ND	2	0	0

[a] A total of 10^3 target cells were incubated with effector cells for 5 h.
[b] D4V antigen-treated target cells were used for D4V-specific clones CB6.1, CB6.3, CB6.15, CB6.21, and CB6.22, and D2V antigen-treated target cells were used for D2V- and D4V-cross-reactive clones CB6.18, CB6.19, and CB6.20.
[c] The effector/target ratios were 7:1 for CB6.1, CB6.15, CB6.22, and CB6.18; 8:1 for CB6.19; 10:1 for CB6.3; 12:1 for CB6.20; and 18:1 for CB6.21 in experiment 1; 10:1 for CB6.1, CB6.15, CB6.21, and CB6.22; and 20:1 for CB6.18, CB6.19, and CB6.20 in experiment 2; 6:1 for CB6.15; 9:1 for CB6.22; 11:1 for CB6.3; 13:1 for CB6.1; 14:1 for CB6.19; 15:1 for CB6.20; and 20:1 for CB6.21 and CB6.19.
[d] ND, no data.

TABLE 3. HLA-DR7-restricted recognition of D4V antigen by a $CD4^+$ CTL clone (CP5) established from donor 2[a]

Expt and target cells	Shared HLA class II allele(s)	% Specific ^{51}Cr release for CP5[b]
Expt 1		
Autologous	DR5, DR7, DQw2, DQw3, DRw52, DRw53	26
CB	DR7, DQw2, DRw53	50
9049	DR7, DQw2, DRw53	53
PG	DR5, DQw2, DQw3	0
FE	DR5, DQw2, DQw3	0
9038	DQw3 (DQ7)	0
Expt 2		
Autologous	DR5, DR7, DQw2, DQw3, DRw52, DRw53	92
9052	DR7, DQw3 (DQ9), DRw53	68
VAVY	DQw2, DRw52	0
Expt 3		
Autologous	DR5, DR7, DQw2, DQw3, DRw52, DRw53	82
JC	DQw3, DRw53	0
MS	DQw3, DRw53	0
Expt 4		
Autologous	DR5, DR7, DQw2, DQw3, DRw52, DRw53	54
CB	DR7, DQw2, DRw53	90
TB	DR5, DQw2	4
A11	DQw2	0
A2	DQw3	2

[a] A total of 10^3 D4V antigen-treated target cells were incubated with effector cells for 5 h.
[b] The effector/target ratios were 9:1 in experiment 1, 10:1 in experiment 2, and 15:1 in experiments 3 and 4.

4). One D4V-specific clone, CB6.22, and three D2V- and D4V-cross-reactive clones, CB6.18, CB6.19, and CB6.20, did not lyse target cells infected with any of the tested recombinant vaccinia viruses, implying that these clones recognized epitopes not represented in these recombinant constructs.

DISCUSSION

In this paper, we characterized a panel of E protein-specific human $CD4^+$ $CD8^-$ CTL clones. E protein was recognized by human $CD4^+$ CTL clones derived from two D4V-immune individuals. All $CD4^+$ CTL clones which recognized E protein were D4V specific and HLA-DR7 restricted. This is the first demonstration of the presence of human $CD4^+$ T-cell epitope(s) on dengue virus E protein, although the epitopes on E protein recognized by murine helper T cells have been reported by other investigators (25, 28, 30).

These serotype-specific $CD4^+$ T cells may play an important role in protection against secondary infections with a dengue virus of the same serotype. In the absence of complete neutralization of challenge virus in secondary infections, it is probable that monocytes and activated lymphocytes will be infected by homologous dengue virus. These infected cells that express high levels of HLA class II need to be eliminated by dengue virus-specific $CD4^+$ and $CD8^+$ CTLs. We have previously reported the existence of E protein-specific $CD8^+$ CTLs in donor 1 (3). Interleukin-2-producing $CD4^+$ CTLs may be instrumental in enhancing clonal expansion of dengue virus-specific $CD8^+$ CTLs. In addition, E protein-specific $CD4^+$ T lymphocytes provide help necessary for anamnestic neutralizing antibody responses which are predominantly E protein specific (4, 7, 13). Passive transfer of neutralizing antibody can protect mice from lethal infection with dengue viruses (17). Therefore, serotype-specific $CD4^+$ CTLs in conjunction with neutralizing antibody probably mediate the long-term protec-

TABLE 4. Recognition of E protein by D4V-specific $CD4^+$ CTL clones[a]

Expt and antigen with which autologous LCL were infected or cultured[b]	% specific ^{51}Cr release[c]								
	D4V specific clones						D2V- and D4V-cross-reactive clones		
	CB6.1	CB6.3	CB6.15	CB6.21	CB6.22	CP5	CB6.18	CB6.19	CB6.20
Expt 1									
VV(D4:E)	38	21	ND[d]	43	14	69	8	7	5
VV(D4:NS3)	0	1	ND	0	3	4	2	3	0
VV(D2:C-PrM-E)	0	0	ND	0	0	ND	0	0	0
VV(control)	0	5	ND	4	5	11	2	4	1
D4V antigen	58	ND	ND	54	85	36	37	40	44
D2V antigen	0	ND	ND	7	7	ND	27	35	41
None	2	ND	ND	2	5	0	7	2	1
Expt 2									
VV(D4:E)	85	85	75	85	ND	ND	ND	ND	ND
VV(D4:NS1-NS2a)	0	0	0	0	ND	ND	ND	ND	ND
VV(control)	13	3	7	0	ND	ND	ND	ND	ND
Expt 3									
VV(D4:E)	30	14	ND	54	11	50	6	8	7
VV(D4:NS3)	0	0	ND	0	0	5	7	2	0
VV(control)	2	2	ND	2	7	1	6	5	6
D4V antigen	41	ND	ND	48	68	71	21	28	34
None	2	ND	ND	2	5	0	3	3	0

[a] A total of 10^3 target cells were incubated with effector cells for 5 h.
[b] VV(D4:E), recombinant vaccinia virus which contains genes coding for E protein of D4V; VV(D4:NS3), recombinant vaccinia virus which contains genes coding for NS3 protein of D4V; VV(D2:C-PrM-E), recombinant vaccinia virus which contains genes coding for C, PrM, and E proteins of D2V; VV(D4:NS1-NS2a), recombinant vaccinia virus which contains genes coding for NS1 and NS2a proteins of D4V.
[c] The effector/target ratios were 7:1 for CP3; and 20:1 for CB6.1, CB6.3, CB6.21, CB6.22, CB6.18, CB6.19, and CB6.20 in experiment 1; 10:1 for CB6.1, CB6.3, CB6.15, and CB6.21 in experiment 2; and 6:1 for CP3 and 20:1 for CB6.1, CB6.3, CB6.21, CB6.22, CB6.18, CB6.19, and CB6.20 in experiment 3.
[d] ND, no data.

tive immunity against challenge with a homologous serotype of virus.

The observation that most of the serotype-specific $CD4^+$ CTL clones established from these donors recognize epitopes on E protein is of great interest. E protein is a 56- to 57-kDa glycosylated protein with 60 to 70% conservation of amino acid sequence homology among the four serotypes (6, 14, 16). E protein of strains within each serotype share more than 95% sequence homology (6, 16). In contrast, there is an approximately 75% sequence homology among the more highly conserved nonstructural proteins NS3 and NS5 (16). We previously described serotype-cross-reactive $CD4^+$ CTL clones that recognize NS3 protein (18). The relative lack of serotype-cross-reactive, E protein-specific CTL clones isolated from donors 1 and 2 may be secondary to the decreased amino acid conservation among E proteins compared with that of nonstructural proteins. Furthermore, there is compelling evidence that virus-specific memory B cells selectively present antigenic peptides to T cells (5, 26, 27). The recognition sites of B cells and $CD4^+$ T cells are topographically linked on influenza virus hemagglutinin (1, 33). Immunization of mice with dengue virus synthetic peptides induced virus-specific or peptide-specific IgG antibodies, suggesting the presence of a B-cell and helper T-cell epitope on one peptide (28, 30). Both the IgG response and the T-cell response to primary dengue virus infection are predominantly serotype specific, with lower levels of serotype cross-reactivity present (8, 24). Serotype-specific, E protein-specific B cells likely favor selection of serotype-specific, E protein-specific memory $CD4^+$ T cells.

DHF usually occurs during secondary infections with a serotype of dengue virus different from the virus which caused the primary infection (8). We have previously shown that soluble CD4 is significantly increased in the sera of children with DHF relative to children with uncomplicated dengue fever (21). Our group has hypothesized that DHF may be triggered by brisk stimulation of serotype cross-reactive memory T cells and the massive production and release of vasoactive cytokines (19). One source of vasoactive cytokines may be infected monocytes which are lysed by cytotoxic T lymphocytes. Monocytes could be targeted for lysis by E protein-specific $CD4^+$ CTL clones through Fc receptor internalization of antibody-bound envelope proteins (23). Evidence for virus-specific $CD4^+$ CTL clones in a widening range of human and primate virus infections continues to accumulate. Recently, Hammond et al. compared the $CD4^+$ versus $CD8^+$ gp160-specific CTL clones in human immunodeficiency virus type 1 vaccine recipients (12). A role for $CD4^+$ CTL-mediated immunopathology secondary to lysis of uninfected CD4 cells which process free gp160 bound to CD4 receptors was suggested. Penna et al. demonstrated that hepatitis B virus-specific $CD4^+$ CTL clones efficiently lysed autologous HLA class II-positive cells expressing endogenously synthesized envelope antigens (29). Five of our dengue virus serotype-specific $CD4^+$ CTL clones efficiently lysed autologous HLA class II-positive LCL infected with DV-VV expressing D4V E protein. No serotype-cross-reactive clones lysed LCL infected with DV-VV expressing D4V E protein or D2V structural proteins despite efficient lysis of dengue virus antigen-treated LCL. These data suggest that serotype-cross-reactive CTL clones CB6.18, CB6.19, and CB6.20 recognize epitopes on nonstructural proteins not represented by our DV-VV constructs.

All of the $CD4^+$ CTL clones that we isolate from donors 1 and 2 examined were HLA-DR7 restricted. E protein-specific, HLA-DR7-restricted $CD4^+$ CTL clones from donor 1 may recognize the same epitope on E protein, which may be identical to or different from the epitope recognized by donor 2. Other HLA haplotypes may favor expansion of other CTL subsets, including serotype-cross-reactive, E protein-specific $CD4^+$ CTL clones. We plan to define the epitope(s) on E protein at the amino acid level using recombinant vaccinia viruses containing truncated D4V E protein genes, as well as synthetic peptides. Identification of epitopes recognized by $CD4^+$ CTL clones from these and other donors should provide important insights for understanding human T-cell responses to dengue viruses and the pathogenesis of DHF.

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