JOURNAL OF VIROLOGY, May 1998, p. 3999–4004 Vol. 72, No. 5
0022-538X/98/$04.00+0

Predominance of HLA-Restricted Cytotoxic T-Lymphocyte Responses to Serotype-Cross-Reactive Epitopes on Nonstructural Proteins following Natural Secondary Dengue Virus Infection

ANUJA MATHEW,[1] ICHIRO KURANE,[1]† SHARONE GREEN,[1] HENRY A. F. STEPHENS,[2] DAVID W. VAUGHN,[3] SIRIPEN KALAYANAROOJ,[4] SAROJ SUNTAYAKORN,[5] DASNAYANEE CHANDANAYINGYONG,[6] FRANCIS A. ENNIS,[1] AND ALAN L. ROTHMAN[1]*

Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical Center, Worcester, Massachusetts[1]; Division of Nephrology, The Middlesex Hospital, London, United Kingdom[2]; and Department of Virology, Armed Forces Research Institute of Medical Sciences,[3] Queen Sirikit National Institute for Child Health (formerly Bangkok Children's Hospital),[4] and Department of Transfusion Medicine, Siriraj Hospital,[6] Bangkok, and Kamphaeng Phet Provincial Hospital, Kamphaeng Phet,[5] Thailand

Received 23 December 1997/Accepted 9 February 1998

We examined the memory cytotoxic T-lymphocytic (CTL) responses of peripheral blood mononuclear cells (PBMC) obtained from patients in Thailand 12 months after natural symptomatic secondary dengue virus infection. In all four patients analyzed, CTLs were detected in bulk culture PBMC against nonstructural dengue virus proteins. Numerous $CD4^+$ and $CD8^+$ CTL lines were generated from the bulk cultures of two patients, KPP94-037 and KPP94-024, which were specific for NS1.2a (NS1 and NS2a collectively) and NS3 proteins, respectively. All CTL lines derived from both patients were cross-reactive with other serotypes of dengue virus. The $CD8^+$ NS1.2a-specific lines from patient KPP94-037 were HLA B57 restricted, and the $CD8^+$ NS3-specific lines from patient KPP94-024 were HLA B7 restricted. The $CD4^+$ CTL lines from patient KPP94-037 were HLA DR7 restricted. A majority of the $CD8^+$ CTLs isolated from patient KPP94-024 were found to recognize amino acids 221 to 232 on NS3. These results demonstrate that in Thai patients after symptomatic secondary natural dengue infections, CTLs are mainly directed against nonstructural proteins and are broadly cross-reactive.

Dengue hemorrhagic fever (DHF), the severe form of dengue illness, is hypothesized to be the result of the overactivation of the immune system (15). In its most serious form, dengue shock syndrome (DSS), the plasma leakage of DHF leads to shock and can be life threatening. Dengue virus infections are commonly seen in South Asia, Southeast Asia, the Caribbean, and South America. In the United States, the disease is seen in residents returning from travel to tropical countries. Antibodies to one serotype of dengue virus have been shown to enhance infection with another serotype of dengue virus in vitro by a process known as antibody-dependent enhancement (9). Epidemiological observations suggest that antibodies capable of mediating antibody-dependent enhancement in vitro are a risk factor for developing DHF or DSS and that complications of dengue illness are more commonly seen in secondary infections (20). These observations suggest that vaccines against dengue should induce protection against all four serotypes.

Our laboratory has been involved in the understanding of the human $CD4^+$ and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses to dengue virus infection. We have identified $CD4^+$ and $CD8^+$ T-cell responses to several proteins of dengue virus in previously unexposed healthy Caucasians who received live candidate monovalent dengue virus vaccines (6, 19). These studies have shown a predominance of recognition of serotype-cross-reactive epitopes on the nonstructural proteins NS3 and NS1.2a (NS1 and NS2a collectively), with less prominent recognition of serotype-specific epitopes on structural proteins. We hypothesize that the cross-reactive T cells from the primary dengue virus infection are reactivated during a secondary infection and play a role in recovery from and/or immunopathology of infection.

We therefore examined dengue virus-specific CTL responses in peripheral blood mononuclear cells (PBMC) of four children 12 months after a secondary natural infection obtained as part of a prospective study of dengue virus infections in Thailand (10). In all four patients, CTLs were detected in bulk culture against at least one protein of dengue virus. In two of these patients, limiting-dilution cloning experiments generated numerous $CD4^+$ and $CD8^+$ CTL lines directed against serotype-cross-reactive epitopes on the nonstructural proteins NS1.2a and NS3. These results are consistent with our hypothesis that cross-reactive cells from the primary infection are reactivated after a secondary infection. This is the first report of dengue virus-specific CTL responses after natural secondary dengue virus infections.

MATERIALS AND METHODS

Viruses. Dengue 2 (D2) virus (New Guinea C strain) was provided by Walter E. Brandt (Walter Reed Army Institute of Research). Dengue 4 (D4) virus (Caribbean strain 814669) was provided by Jack McCown (Walter Reed Army Institute of Research). Viruses were propagated as previously described (13) and frozen at −70°C until use. Recombinant vaccinia viruses containing genes coding for dengue virus proteins produced as described previously (Vac.D2NS3 [vaccinia virus expressing D2 virus NS3], etc.) were kindly provided by C. J. Lai (National Institutes of Health, Bethesda, Md.), Enzo Paoletti (Virogenetics

* Corresponding author. Mailing address: Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-4182. Fax: (508) 856-4890. E-mail: Alan.Rothman@ummed.edu.

† Present address: Department of Microbiology, Kinki University School of Medicine, Osaka, Japan.

TABLE 1. Clinical, viral, and immunogenetic profiles of the study subjects

Patient	Disease	Viral serotype	HLA class I alleles			HLA class II alleles		
			A	B	C	DR	DQ	DP
KPP94-037	Dengue fever	D2	1,11.1	46,57	1,6	DRB1 *0701 DRB1 *0901 DRB4 *0101	DQA1 *0201 DQA1 *03011 DQB1 *03032	DPB1 *0501 DPB1 *1301
KPP94-024	DHF grade 3	D2	2,24	7,46	1,7	DRB1 *0901 DRB1 *1201 DRB3 *0301 DRB4 *0101	DQA1 *03011 DQA1 *0102 DQB1 *03032 DQB1 *0502	DPB1 *0501 DPB1 *1301
CHD94-134	DHF grade 3	D2	11.1,28	27,57	3,6	DRB1 *0701 DRB1 *1202 DRB3 *0301	DQA1 *0201 DQA1 *0601 DQB1 *0301 DQB1 *03032	DPB1 *0401 DPB1 *1301
CHD94-020	DHF grade 2	D4	2,11.1	7,46	1,7	DRB1 *0901 DRB1 *1110 DRB3 *0202 DRB4 *0101	DQA1 *03011 DQA1 *05011 DQB1 *0301 DQB1 *03032	DPB1 *0501 DPB1 *1301

Corporation, Troy, N.Y.), and Margo A. Brinton (Georgia State University, Atlanta) (5, 22, 23).

Study protocol and PBMC. Blood samples were obtained from children enrolled in a prospective study of dengue virus infections at the Queen Sirikit National Institute for Child Health (formerly Bangkok Children's Hospital), Bangkok, Thailand, and the Kamphaeng Phet Provincial Hospital, Kamphaeng Phet, Thailand, in 1994 (10). Children with fever of 72 h or less in duration without an obvious cause and facial flushing were eligible to participate in this study. A diagnosis of acute dengue virus infection was based on serologic tests (antibody capture enzyme immunoassay and hemagglutination inhibition) and isolation of dengue virus (in *Toxorhynchites splendens* mosquitoes) (21). A diagnosis of secondary dengue virus infection was made on the basis of (i) a dengue virus immunoglobulin M (IgM)-to-IgG ratio of $<1.8:1$; (ii) with serial specimens, a twofold increase in IgG to dengue virus, with an absolute value of ≥ 100 U in the absence of IgM to dengue virus of ≥ 40 U; and (iii) a hemagglutination inhibition titer of $>1:1,280$ 1 week after the onset of illness. Written consent was obtained from each subject's parents or guardians. The study protocol was approved by the Institutional Review Boards established by the Ministry of Health, Thailand, the Surgeon General's Office of the Department of the Army, and the University of Massachusetts Medical Center. PBMC obtained 12 months after dengue virus infection were separated and cryopreserved at -70°C until further use. Frozen PBMC samples were shipped to the University of Massachusetts Medical Center for testing. HLA-A, -B, and -C class I typing was performed by using a standard microlymphocytotoxicity assay, and HLA class II typing was performed by PCR-based amplification and hybridization with HLA allele DRB1, DRB3, DRB5, DQA1, DQB1, and DPB1 sequence-specific oligonucleotide probes as previously described (3). Four subjects with documented secondary D2 or D4 virus infections were selected for this study (Table 1).

Bulk culture of PBMC. PBMC were initially cultured with 0.25 ml containing approximately 10^7 focus-forming units of the homologous infecting serotype of dengue virus in 0.75 ml of AIM-V medium (GIBCO BRL Life Technologies, Gaithersburg, Md.) containing 10% heat-inactivated human AB serum (Hu ABS; Advanced Biotechnologies, Inc., Columbia, Md.). PBMC were then restimulated 7 to 9 days later with 10^6 gamma-irradiated (3,500 Rad) allogeneic PBMC and anti-CD3 monoclonal antibody 12F6 (0.1 μg/ml), kindly provided by Johnson Wong, in 0.5 ml of fresh medium containing 10% Hu ABS and 25 to 50 U of recombinant interleukin-2 (IL-2; Collaborative Biochemical Products, Bedford, Mass.) per ml as indicated previously (24). Bulk cultures were restimulated every 2 weeks. Cells were assayed 7 to 10 days after the last restimulation for cytolytic activity in CTL assays.

Cloning of PBMC. PBMC which had been stimulated in bulk culture for 7 days were collected and plated at a concentration of 10 and 30 cells per well in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.) in 200 μl of AIM-V medium containing 10% Hu ABS, 10^5 allogeneic gamma-irradiated PBMC, anti-CD3 (0.1 μg/ml), and recombinant IL-2 (25 U/ml) (24). Every 3 to 4 days, cells were fed with fresh AIM-V medium containing 10% Hu ABS and IL-2 (25 U/ml). Cells were restimulated every 2 weeks. The T-cell lines were initially screened with vaccinia virus recombinants expressing dengue virus proteins. Growing cells that showed positive lytic activity against any dengue virus proteins were expanded into 48-well plates (Costar) and restimulated with 10^6 allogeneic PBMC and anti-CD3 at a final volume of 1 ml.

Preparation of target cells. B-lymphoblastoid cell lines (BLCLs) were established by culturing PBMC with Epstein-Barr virus in 48-well plates (7). Allogeneic BLCLs were also obtained from the National Institutes of General Medical Sciences Human Genetic Mutant Cell Repository or the American Society for Histocompatibility and Immunogenetics Cell Bank and Repository. BLCLs (3×10^5 to 5×10^5) were infected with vaccinia viruses for 1.5 to 2 h at 37°C and then diluted in 2 ml of RPMI 1640–10% fetal bovine serum medium (Sigma Immunochemicals, St. Louis, Mo.) and further incubated for 12 to 16 h. Target cells were labeled with 0.25 mCi of ^{51}Cr for 60 min at 37°C. After three washes, the target cells were counted and diluted to 10^4 cells/ml for use in cytotoxicity assays.

^{51}Cr release cytotoxicity assay. Cytotoxicity assays were performed in 96 round-bottom wells as previously reported (2, 19). Effector cells were added to 10^3 ^{51}Cr-labeled target cells at various effector-to-target (E/T) ratios. In CTL assays with synthetic peptides, peptides were added at 25 μg/ml to target cells and incubated at 37°C for 30 min, after which effector cells were added. Plates were centrifuged at $200 \times g$ for 5 min and incubated 4 to 5 h at 37°C. Supernatant fluids were harvested by using the Skatron Instruments (Sterling, Va.) supernatant collection system, and ^{51}Cr content was measured in a gamma counter. The percent specific ^{51}Cr release was calculated as $100 \times$ (cpm experimental release − cpm spontaneous release)/(cpm maximum release − cpm spontaneous release). All assays were performed in triplicate, and the results were calculated from the average of the triplicate wells. The standard error of the mean was $<10\%$ in all experiments.

RESULTS

Protein specificity of CTLs generated from convalescent PBMC of children after natural secondary dengue virus infection in Thailand. To detect dengue virus-specific CTL, we stimulated convalescent PBMC with the homologous dengue virus and tested for cytolytic activity in bulk culture against the homologous dengue virus proteins. We detected CTL activity in bulk culture PBMC from patient KPP94-037 against target cells infected with Vac.D2NS1.2a and to those infected with Vac.D2NS3 to a lesser degree (Table 2). For patients KPP94-024 and CHD94-020, the predominant CTL response was directed against the homologous NS3 protein (Table 2). For patient CHD94-134, low-level CTL activity was detected in bulk culture against all dengue virus-vaccinia virus recombinants tested. These results show that in bulk culture experiments, PBMC of all four patients had detectable cytolytic activity against target cells expressing nonstructural proteins NS1.2a and/or NS3.

Cross-reactivity of T-cell lines established from PBMC of patients KPP94-037 and KPP94-024. T-cell lines were established by limiting dilution from the bulk cultures obtained from patients KPP94-037 and KPP94-024. For patient KPP94-037, the lines were initially screened against Vac.control, Vac.D2NS3, and Vac.D2NS1.2a. All lines that had positive lytic activity were found to be directed against the nonstructural proteins NS1.2a (data not shown). Among 12 T-cell lines, four lines had the $CD3^+$ $CD4^+$ $CD8^-$ phenotype and eight lines had the $CD3^+$ $CD4^-$ $CD8^+$ phenotype (Table 3). The

TABLE 2. Bulk culture CTL of PBMC obtained from children 1 year after natural infection[a]

Target cells infected with:	% Dengue virus-specific ^{51}Cr release[b]			
	KPP94-037	KPP94-024	CHD94-020	CHD94-134
Vac.D2CprME	0	−11		15
Vac.D2NS1.2a	38	5		16
Vac.D2NS3	13	38		18
Vac.D4CprME			−12	
Vac.D4NS1.2a			−6	
Vac.D4NS3			29	

[a] PBMC (1×10^6 to 3×10^6) were stimulated in vitro with the homologous serotype of dengue virus (D2 for KPP94-037, KPP94-024, and CHD94-134 and D4 for CHD94-020). Cells were restimulated with anti-CD3 and allogeneic feeders on day 7 and then every 2 weeks. The cells were used as effectors and tested in CTL assays on days 14 to 35 of culture.

[b] Represents lysis of target cells infected with recombinant viruses − lysis by Vac.control. The Vac.control values were 6% for KPP94-037, 51% for KPP94-024, 29% for CHD94-020; and 33% for CHD94-134. E/T ratios were 70:1 for KPP94-037 and 50:1 for other donors.

TABLE 4. Cross-reactivity of CTL lines generated from PBMC of patient KPP94-024 1 year after dengue virus infection[a]

Line	Phenotype	% Specific ^{51}Cr release[b]			
		Vac.control	Vac.D2NS3	Vac.D3NS3	Vac.D4NS3
2B2	CD4$^+$	1	80	82	76
2D11	CD4$^+$	0	53	40	29
2C9	CD4$^+$	−1	81	75	72
3E5	CD4$^+$	0	72	72	68
2B11	CD4$^+$	0	68	65	63
2E3	CD8$^+$	0	80	75	76
3C3	CD8$^+$	−3	68	82	77
3B11	CD8$^+$	0	76	73	89
2G10	CD8$^+$	0	63	64	61
2C8	CD8$^+$	2	84	88	84
3C2	CD8$^+$	2	77	77	83
2F5	CD8$^+$	−2	86	77	78

[a] T-cell lines were established from bulk culture of PBMC from patient KPP94-024 by the limiting-dilution method. The phenotype of the cells was determined by fluorescent antibody staining.

[b] Measured at E/T ratios between 20:1 and 60:1 for all T-cell lines used.

NS1.2a-specific CTL lines were tested for cross-reactivity against Vac.D1ENS1.2a and Vac.D4NS1.2a constructs. Recognition of NS1.2a by all the T-cell lines was cross-reactive to D1, D2, and D4 viruses (Table 3).

For patient KPP94-024, 12 NS3-specific T-cell lines were similarly isolated after initial screening against target cells infected with Vac.control and Vac.D2NS3 (data not shown). These T-cell lines were then tested for cross-reactivity to other dengue virus serotypes (Table 4). All of the CTL lines were cross-reactive for D3 and D4 viruses. These results indicate that all the dengue virus-specific T-cell lines established from patient KPP94-037 were serotype cross-reactive and recognized the NS1.2a proteins and that those established from PBMC of patient KPP94-024 were also serotype cross-reactive but recognized the NS3 protein. Protein recognition of T-cell lines from donors KPP94-037 and KPP94-024 were consistent with the recognition of bulk culture CTLs shown in Table 2.

HLA restriction of the lysis of target cells by CD8$^+$ and CD4$^+$ CTL lines from PBMC of patients KPP94-037 and KPP94-024. For patient KPP94-037, allogeneic BLCLs which had HLA alleles in common with autologous cells were infected with Vac.D2NS1.2a and used as targets. Table 5, experiment 1, shows that three representative CD8$^+$ lines were HLA B57 restricted because only targets having B57 in common with the autologous BLCLs were lysed efficiently by CD8$^+$ CTLs. Experiments 2 and 3, using allogeneic BLCLs that had class II alleles in common with the autologous line, indicate that two of the CD4$^+$ CTL lines from patient KPP94-037 were HLA DR7 restricted. For patient KPP94-024, allogeneic BLCLs sharing common class I alleles with autologous BLCLs were infected with D2NS3. Four representative CD8$^+$ lines generated from patient KPP94-024 were B7 restricted (Table 5, experiments 4 and 5). All CD8$^+$ lines established from patient KPP94-037 were B57 restricted, and all lines established from patient KPP94-024 were B7 restricted (data not shown).

TABLE 3. Cross-reactivity of T-cell lines generated from PBMC of patient KPP94-037 1 year after dengue virus infection[a]

Line	Phenotype	% Specific ^{51}Cr release[b]			
		Vac.control	Vac.D1ENS1.2a	Vac.D2NS1.2a	Vac.D4NS1.2a
2G3	CD4$^+$	0	57	91	64
2E8	CD4$^+$	5	59	81	67
2D11	CD4$^+$	5	64	98	73
2B5	CD4$^+$	9	59	93	73
3C11	CD8$^+$	9	56	80	59
3E7	CD8$^+$	8	57	97	100
3F3	CD8$^+$	8	65	100	57
2C8	CD8$^+$	9	63	89	78
2G7	CD8$^+$	4	56	77	58
2D9	CD8$^+$	5	58	96	65
3F11	CD8$^+$	3	51	67	40
3G10	CD8$^+$	4	66	80	59

[a] T-cell lines were established from bulk culture of PBMC from patient KPP94-037 by the limiting-dilution method. The phenotype of the cells was determined by fluorescent antibody staining.

[b] Measured at E/T ratios between 20:1 and 60:1 for all T-cell lines used.

Cross-reactivity, HLA restriction, and epitope analysis of bulk culture CTL generated from PBMC of patient CHD94-020. For patient CHD94-020, bulk culture CTLs lysed targets expressing the NS3 proteins of D2, D3, and D4 viruses (Tables 2 and 6). Using recombinant vaccinia viruses expressing truncated D3NS3, we were able to localize a CTL epitope to amino acids (aa) 1 to 176 of NS3 (Table 6, experiment 2). Using allogeneic targets having HLA class I alleles in common with CHD94-020, we found bulk culture CTL activity to be HLA A11.1 restricted, based on recognition of CHD94-134 BLCL target cells, which share only A11.1 in common with the autologous line (Table 6, experiment 3). These results indicate that the NS3-specific CTLs detected in bulk culture from this subject are A11.1 restricted and are also serotype cross-reactive. We tried to isolate CTL lines from this donor by the limiting-dilution method but were not successful.

Localization of the epitope within the NS3 protein recognized by T-cell lines from PBMC of patient KPP94-024. CTL lines from patient KPP94-024 lysed target cells expressing NS3; therefore, we infected target cells with vaccinia virus recombinants expressing truncations of the D3NS3 gene to localize the epitopes recognized by these CTL lines. We mapped the region on the NS3 protein recognized by all T-cell lines tested from patient KPP94-024 to aa 216 to 247 of NS3 (Table 7, experiment 1). We then checked for CTL activity against overlapping peptides which spanned that region and found lytic activity only against peptide 221, which is a 15-mer corresponding to aa 221 to 235 (LAPTRVVAAEMEEAL) (data not shown). We used synthetic peptides with truncations of peptide 221 to detect the minimum epitope recognized by the

TABLE 5. HLA restriction of recognition of dengue virus proteins by CTL lines of patients KPP94-037 and KPP94-024[a]

Patient KPP94-037[b]															Patient KPP94-024[c]										
Expt 1					Expt 2					Expt 3					Expt 4					Expt 5				
BLCL	HLA class I	% Specific ^{51}Cr release by indicated T-cell line			BLCL	HLA class II	% Specific ^{51}Cr release by indicated T-cell line		BLCL	HLA class II	% Specific ^{51}Cr release by indicated T-cell line		BLCL	HLA class I	% Specific ^{51}Cr release by indicated T-cell line		BLCL	HLA class I	% Specific ^{51}Cr release by indicated T-cell line						
		3E7	2E4	2C8			2D11	2G3			2D11	2G2			2F5	2C8			2F5	3C2	3B11				
Autologous	A1,11.1 B46,57 C1,6	95	91	79	Autologous	DP5 DQ9 DR7,9	56	55	Autologous	DP5 DQ9 DR7,9	71	39	Autologous	A2,24 B7,46 C1,7	70	68	Autologous	A2,24 B7,46 C1,7	78	75	82				
HCV57	A2,1,3 B7,37 C6,7	11	−4	−4	9077	DP5 DQ3 DR9,12	−3	−7	KPP94-024	DP5 DQ9 DR9,12	−3	1	VA03	A2,24 B7,35 C3,7	32	23	Tom G	A23,29 B7,44 C4	28	56	51				
9052	A1,3 B27,57 C2,6	29	22	21	CP	DP4 DQ2,3 DR5,7	30	30	CHD94-134	DP4 DQ9,7 DR7,12	78	34	9077	A2 B46 C11	−5	−5	GM3161	A3,3 B7,7	41	44	46				
GM3098A	A2,2 B 57,57	71	51	53					MS	DQ1,3 DR1,4		−7	CP	A2,28 B51,57 C6	−4	0	GM6825A	A23 B7,7	26	53	52				

[a] Allogeneic BLCLs that shared one or more HLA class I or class II alleles with the autologous line, as indicated, were used as targets in the assays. The E/T ratios were between 16:1 and 40:1 for all T-cell lines used.
[b] Target cells were infected with Vac.D2NS1.2a.
[c] Target cells were infected with Vac.D2NS3.

TABLE 6. Cross-reactivity, epitope localization, and HLA restriction of bulk culture CTL obtained from PBMC of patient CHD94-020[a]

Expt	Target cells infected with:	% Specific ^{51}Cr release
1	Vac.control	24
	Vac.D2C.prM.E	25
	Vac.D2NS1.2a	28
	Vac.D2NS3	45
2	Vac.control	17
	Vac.D3NS3	54
	Vac.D3 (aa 1–176)	54
3	Autologous (2,11.1; 7,46; 1,7[b])	49
	KPP94-024 (2,24; 7,46; 1,7)	17
	KPP94-037 (1,11.1; 46,57; 1,6)	56
	CHD94-134 (11.1,28; 27,57; 3,6)	45

[a] PBMC from patient CHD94-020 were stimulated with D4 virus and 7 days later restimulated with anti-CD3 and allogeneic feeders. Cells were restimulated every 2 weeks and tested in CTL assays 7 to 10 days after the last restimulation. The E/T ratios were 50:1 for experiments 1 and 2 and 106:1 for experiment 3.
[b] Alleles A, B, and C, respectively.

T-cell lines (Table 7, experiments 2 and 3). Peptide targets pulsed with truncations 221g and 221f were lysed by most CTL, whereas target cells pulsed with truncations 221a, 221c, and 221d were not lysed. T-cell lines 2F5 and 3C3 recognized aa 222 to 230 of NS3, and most of the other T-cell lines recognized targets that were pulsed with aa 221 to 232 of NS3.

DISCUSSION

Most of the research performed on human T-cell cytotoxic responses to dengue viruses has been in Caucasian volunteers who received experimental live monovalent dengue virus vaccines. These individuals had no known prior exposure to dengue viruses and therefore received the dengue virus vaccine as a primary infection. However, the complications seen in dengue virus infection (DHF and DSS) are more common in patients who have preexisting immunity to one serotype of virus during infection with another serotype (secondary infection) of virus (8). Virus-specific memory T cells have been implicated in contributing to the pathogenesis of severe dengue virus infection (16). Analysis of T-cell responses in patients after natural secondary infections is therefore important because it may provide insights into the mechanisms of T-cell-mediated immunopathology. In this study, memory CTL responses were detected against dengue virus proteins in the PBMC of all four patients examined. This is the first report of dengue virus-specific CTLs after natural secondary dengue virus infections.

We have previously shown that nonstructural proteins, in particular NS3, are predominant targets for both $CD4^+$ and $CD8^+$ CTLs in vaccine recipients (17, 19, 22). In the present study, we also found that three of four donors had CTL responses to NS3 and that the fourth donor had CTLs which recognized the nonstructural proteins NS1.2a. Proteins with a cytoplasmic intracellular localization have been noted to be the predominant source of cytotoxic T-cell antigenic peptides (18). The NS3 protein constitutes approximately 25% of the cytoplasmic region of the polyprotein, which may explain why it is an immunodominant target for CTL recognition. Numerous other factors (rate of proteolysis, efficiency of transport, peptide stability, etc.) are also likely to influence why peptides from nonstructural proteins are preferentially presented on major histocompatibility complex molecules (1). Since we have

TABLE 7. Localization of the epitope on NS3 recognized by CTL lines of patient KPP94-024[a]

Expt	Target cells infected/ pulsed with:	% Specific ^{51}Cr release							
		2G10	2F5	3C2	3C3	2E4	3E5	3B11	2C9
1[b]	Vac.control	ND	ND	2	−7	−3	−5	−1	−1
	Vac.D3NS3			70	63	64	37	62	55
	Vac.D3NS3 (aa 1–133)			−2	−5	−2	−4	−4	−6
	Vac.D3NS3 (aa 1–176)			−4	0	−2	−2	−2	−3
	Vac.D3NS3 (aa 1–216)			11	−3	2	−2	3	0
	Vac.D3NS3 (aa 1–247)			63	71	59	37	61	61
2[c]	No peptide	2	ND	0	ND	1	ND	ND	ND
	D4NS3 221 (aa 221–235)	21		50		70			
	D4NS3221a (aa 224–235)	2		1		1			
	D4NS3 221c (aa 226–235)	−1		1		1			
	D4NS3 221d (aa 227–235)	−1		1		2			
	D4NS3 221f (aa 221–233)	12		35		50			
	D4NS3 221g (aa 221–232)	5		24		58			
3[c]	No peptide	ND	1	ND	−5	0	ND	1	3
	D4NS3 221 (aa 221–235)		61		53	48		39	33
	D4NS3 221g (aa 221–232)		47			34		52	22
	D4NS3 222z (aa 222-230)		30		33	18		11	9

[a] The E/T ratios were between 10:1 and 60:1 for all T-cell lines used. ND, not determined.
[b] BLCLs were infected with vaccinia virus recombinants expressing different truncations of the NS3 protein as indicated and used as target cells.
[c] BLCLs were pulsed with 25 μg of the indicated peptide per ml for 30 min, after which the T cells were added and the sample was further incubated for 4 to 5 h.

not tested vaccinia virus recombinants expressing NS5, it is possible that there are also epitopes on NS5.

Immune responsiveness is affected by the major histocompatibility complex, and the pathogenesis of some diseases has been associated with specific HLA alleles. In areas where DHF is endemic, only a small percentage of individuals exhibit severe disease, which suggests that host genetic factors may play a role in susceptibility to severe disease. One study found a positive association between HLA A2 and B blank and the development of DSS and a negative relationship for HLA B13 (4).

Since it is known that there is diversity in both HLA and non-HLA gene loci between Southeast Asians and Caucasians (3), it was important to analyze the recognition of dengue virus proteins by T cells in the context of HLA molecules in Thai patients. We previously isolated NS3-specific $CD4^+$ and $CD8^+$ CTL clones from Caucasian volunteers which were restricted by the HLA alleles B35 (17), DR15 (12), and DPw2 (14). The NS1.2a-specific CTLs from patient KPP94-037 in the present study were B57 restricted and DR7 restricted; the NS3-specific CTLs from patient KPP94-024 were B7 restricted, and the NS3-specific CTLs from patient CHD94-020 were A11.1 restricted. These results indicate that despite differences in HLA alleles between Thais and Caucasians, peptides from dengue virus nonstructural proteins are also predominantly recognized by T cells from Thai children. These studies confirm our previous results of the dominance of nonstructural proteins as CTL targets in Caucasian dengue virus vaccine recipients. All of the T-cell lines isolated from patient KPP94-024 recognized autologous targets pulsed with aa 221 to 235 of the NS3 protein. The 15-mer which is lysed by all the B7 restricted $CD8^+$ CTLs from this patient contains a proline at position 223. These results are entirely consistent with reports of antigenic peptides bearing a proline at position 2 and aromatic or hydrophobic residues at the C terminus, preferentially binding to HLA B7 and related class I alleles sharing the B7-like supertype (21).

Our earlier work analyzing primary responses from PBMC of vaccine recipients, detected both serotype-specific and serotype-cross-reactive CTL responses against many proteins in individual donors (11, 19). We speculated that the serotype-cross-reactive memory T cells generated during the primary infection would be reactivated in a secondary infection and contribute to the immunopathology of DHF which is observed much more frequently in secondary infections. In this study, we have examined CTL responses after secondary infection in four Thai patients. All the NS1.2a-specific CTL lines generated from patient KPP94-037 and the NS3-specific CTLs from patient KPP94-024 in this study were cross-reactive with the other serotypes of dengue virus. The bulk culture CTLs from patient CHD94-020 were also cross-reactive with D2 and D3 virus NS3. We have not yet analyzed the primary CTL responses in Thai children; therefore, we cannot conclusively define the relationship between primary and secondary dengue virus infections. However, these results suggest that the CTL responses in secondary infections are predominantly due to reactivation of memory cross-reactive CTL from the primary infections, consistent with our hypothesis.

In conclusion, the CTL responses of Thai patients to natural secondary dengue virus infection are directed against nonstructural proteins and are mainly cross-reactive in nature.

ACKNOWLEDGMENTS

We thank Jurand Janus for preparation of viral antigens.

This work was supported by grants NIH P01-AI34533 and NIH R01-AI30624 from the National Institutes of Health.

REFERENCES

1. **Barber, L. D., and P. Parham.** 1994. The essence of epitopes. J. Exp. Med. **180:**1191–1194.
2. **Bukowski, J. F., I. Kurane, C.-J. Lai, M. Bray, B. Falgout, and F. A. Ennis.** 1989. Dengue virus-specific cross reactive $CD8^+$ human cytotoxic T lymphocytes. J. Virol. **63:**5086–5091.
3. **Chandanayingyong, D., H. A. F. Stephens, R. Klaythong, M. Sirikong, S. Udee, P. Longta, R. Chntangpol, S. Bejrachandra, and E. Rungruang.** 1997. HLA-A, -DRB1, -DQA1 and -DQB1 polymorphism in Thais. Hum. Immunol. **53:**174–182.
4. **Chiewslip, P., R. Scott, and N. Bhamarapravati.** 1981. Histocompatibility antigens and dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. **30:**1101–1105.

5. **Falgout, B., R. Chanock, and C.-J. Lai.** 1989. Proper processing of dengue virus nonstructural protein NS1 requires N terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. J. Virol. **63:**1852.
6. **Gagnon, S. J., W. Zeng, I. Kurane, and F. A. Ennis.** 1996. Identification of two epitopes on the dengue 4 virus capsid protein recognized by a serotype specific and a panel of serotype cross-reactive human $CD4^+$ cytotoxic T-lymphocyte clones. J. Virol. **70:**141–147.
7. **Green, S., I. Kurane, R. Edelman, C. O. Tacket, K. H. Eckles, D. W. Vaughn, C. H. Hoke, and F. A. Ennis.** 1993. Dengue virus-specific human $CD4^+$ T-lymphocyte responses in a recipient of an experimental live-attenuated dengue virus type 1 vaccine: bulk culture proliferation, clonal analysis, and precursor frequency determination. J. Virol. **67:**5962–5967.
8. **Halstead, S. B.** 1988. Immunological parameters of togavirus disease syndromes, p. 107–173. *In* R. W. Schlesinger (ed.), The togaviruses: biology, structure, replication. Academic Press, New York, N.Y.
9. **Halstead, S. B., S. Nimmannitya, and S. N. Cohen.** 1970. Observations related to the pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J. Biol. Med. **42:**311–328.
10. **Kalyanarooj, S., D. W. Vaughn, S. Nimmannitya, S. Green, S. Suntayakorn, N. Kunentrasai, W. Viramitrachai, S. Ratanachu-eke, S. Kiatpolpoj, B. L. Innis, A. L. Rothman, A. Nisalak, and F. A. Ennis.** 1997. Early clinical and laboratory indicators of acute dengue illness. J. Infect. Dis. **176:**313–321.
11. **Kurane, I., M. A. Brinton, A. L. Samson, and F. A. Ennis.** 1991. Dengue virus-specific, human $CD4^+$ $CD8^-$ cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. J. Virol. **65:** 1823–1828.
12. **Kurane, I., M. A. Brinton, A. L. Samson, and F. A. Ennis.** 1991. Dengue virus-specific, human $CD4^+$ $CD8^-$ cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. J. Virol. **65:** 1823–1828.
13. **Kurane, I., D. Hebblewaite, Walter E. Brandt, and F. A. Ennis.** 1984. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. J. Virol. **52:**223.
14. **Kurane, I., L. Dai, P. G. Livingston, E. Reed, and F. A. Ennis.** 1993. Definition of an HLA-DPw2-restricted epitope on NS3 recognized by a dengue virus serotype-cross-reactive human $CD4^+$ $CD8^-$ cytotoxic T-cell clone. J. Virol. **67:**6285–6288.
15. **Kurane, I., and F. A. Ennis.** 1992. Immunity and immunopathology in dengue virus infections. Semin. Immunol. **4:**121–127.
16. **Kurane, I., B. L. Innis, S. Nimmannitya, A. Nisalak, A. Meager, J. Janus, and F. A. Ennis.** 1991. Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. J. Clin. Invest. **88:**1473–1480.
17. **Livingston, P. G., I. Kurane, L. Dai, Y. Okamoto, C.-J. Lai, R. Men, S. Karaki, M. Takiguchi, and F. A. Ennis.** 1995. Dengue virus-specific, HLA B-35 restricted, human $CD8^+$ cytotoxic T lymphocyte (CTL) clones: recognition of NS3 amino acids 500-508 by CTL clones of two different specificities. J. Immunol. **154:**1287–1295.
18. **Lobigs, M., C. E. Arthur, A. Mullbacher, and R. V. Blanden.** 1994. The flavivirus nonstructural protein NS3 is a dominant source of cytotoxic T cell peptide determinants. Virology **202:**195–201.
19. **Mathew, A., I. Kurane, A. L. Rothman, L. Zeng, M. A. Brinton, and F. A. Ennis.** 1996. Dominant recognition by human $CD8^+$ cytotoxic T lymphocytes of dengue virus nonstructural proteins NS3 and NS1.2a. J. Clin. Invest. **98:** 1684–1692.
20. **Sangdawibha, N., S. Rojanasuphot, S. Ahandrik, S. Viriyaponges, S. Jatanasen, V. Salitul, B. Phanthumachinda, and S. B. Halstead.** 1984. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. Am. J. Epidemiol. **120:**653–669.
21. **Sidney, J., H. M. Grey, R. T. Kubo, and A. Sette.** 1996. Practical, biochemical and evolutionary implications of the discovery of HLA class I supermotifs. Immunol. Today **17:**261–266.
22. **Zeng, L., I. Kurane, Y. Okamoto, F. A. Ennis, and M. A. Brinton.** 1996. Identification of amino acids involved in recognition by dengue virus NS3-specific, HLA-DR15-restricted cytotoxic $CD4^+$ T-cell clones. J. Virol. **70:** 3108–3117.
23. **Zhao, B., G. Prince, R. Horswood, K. H. Eckels, R. M. Chanock, and C.-J. Lai.** 1989. Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus. J. Virol. **64:**4109.
24. **Zivny, J., I. Kurane, and F. A. Ennis.** 1995. Establishment of dengue virus-specific human $CD4^+$ T lymphocyte clones from Percoll-purified T lymphoblasts by stimulation with monoclonal antibody to CD3. J. Immunol. Methods **188:**165–167.