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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

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We analyzed the CD4$^+$ T-lymphocyte response of a donor who had received an experimental live-attenuated dengue 4 virus (D4V) vaccine. Bulk culture proliferative responses of peripheral blood mononuclear cells (PBMC) to noninfectious dengue virus (DV) antigens showed the highest proliferation to D4V antigen, with lesser, cross-reactive proliferation to D2V antigen. We established CD4$^+$ cytotoxic T-lymphocyte clones (CTL) by stimulation with D4 antigen. Using recombinant baculovirus antigens, we identified seven CTL clones that recognized D4V capsid protein. Six of these CTL clones were cross-reactive between D2 and D4, and one clone was specific for D4. Using synthetic peptides, we found that the D4V-specific CTL clone recognized an epitope between amino acids (aa) 47 and 55 of the capsid protein, while the cross-reactive CTL clones each recognized epitopes in a separate location, between aa 83 and 92, which is conserved between D2V and D4V. This region of the capsid protein induced a variety of CD4$^+$ T-cell responses, as indicated by the fact that six clones which recognized a peptide spanning this region showed heterogeneity in their recognition of truncations of this same peptide. The bulk culture response of the donor's PBMC to the epitope peptide spanning aa 84 to 92 was also examined. Peptides containing this epitope induced proliferation of the donor's PBMC in bulk culture, but peptides not containing the entire epitope did not induce proliferation. Also, PBMC stimulated in bulk culture with noninfectious D4V antigen lysed autologous target cells pulsed with peptides containing aa 84 to 92. These results indicate that this donor exhibits memory CD4$^+$ T-cell responses directed against the DV capsid protein and suggest that the response to the capsid protein is dominant not only in vitro at the clonal level but in bulk culture responses as well. Since previous studies have indicated that the CTL responses to DV infection seem to be directed mainly against the envelope (E) and NS3 proteins, these results are the first to indicate that the DV capsid protein is also a target of the antiviral T-cell response.

Dengue viruses (DV) are divided into four antigenically related serotypes, called DV types 1, 2, 3, and 4 (D1V, D2V, D3V, and D4V) (15). Human DV infections can range from asymptomatic to a severe, life-threatening syndrome known as dengue hemorrhagic fever (DHF) (12). Epidemiological studies in Thailand and Cuba have indicated that DHF is more likely to occur following a secondary infection with a serotype of DV other than that which caused the primary infection (5, 12, 13, 19). Although the pathogenesis of DHF is not understood, it has been hypothesized that this syndrome may be immunopathological in nature (14, 21).

It is likely that in DV infection, as in other viral infections, the virus-specific cytotoxic T-lymphocyte (CTL) responses contribute to clearance of the virus through lysis of infected cells and subsequent host recovery. However, it has been demonstrated in several viral systems that virus-specific CTL may also induce immunopathology (2, 6, 30). Thus, we can speculate that the CTL responses to DV infection may contribute to the immunopathology of DHF. In particular, it is possible that DV serotype-cross-reactive CTL generated during the primary infection become activated upon secondary infection, and the resulting high levels of T-cell activation may have a role in the observed pathology.

Our laboratory has examined both the CD4$^+$ and CD8$^+$ CTL responses to various serotypes of DV in order to broaden our understanding of the T-cell response to DV infection and its potential role in both recovery and immunopathology. In bulk culture, human CD8$^+$ CTL from a D4V-immune donor recognized the viral envelope (E) protein as well as one or more of the nonstructural proteins (4). Clonal analysis of CD8$^+$ CTL from this donor showed that both DV serotype-specific and serotype-cross-reactive CTL clones recognize an epitope on NS3, between amino acids (aa) 500 and 508 (26). In the murine system, CD8$^+$ CTL clones were also found to recognize nonstructural proteins, specifically NS3 and either NS1 or NS2a (33). Although CD4$^+$ T cells are generally believed to be of a helper phenotype, our group and others have shown that CD4$^+$ CTL can be generated during an immune response in both mice and humans (1, 7, 25, 35). In DV infection, D4V-specific CD4$^+$ CTL clones recognize E protein (27), and clones of various serotype reactivities from a D3V-immune donor recognize NS3 (20). In short, those limited studies seemed to indicate that the CTL response to DV infection is primarily directed against E and NS3.

In this study, we examined the CD4$^+$ T-cell response of a donor who had received an experimental live-attenuated D4V vaccine. We established CD4$^+$ CTL clones from peripheral blood mononuclear cells (PBMC) collected approximately 6

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months postvaccination. A number of the $CD4^+$ CTL clones were found to recognize the D4V capsid (C) protein. To our knowledge, flavivirus-specific CTL clones recognizing this structural protein have not previously been reported. Six clones that were cross-reactive between D2V and D4V (D2V/D4V cross-reactive) recognized one region of the capsid protein, while one D4V-specific clone recognized a separate region. Further, the peptide epitope recognized by the D2V/D4V cross-reactive clones was able to induce both proliferation and CTL killing at the bulk culture level. These results suggest that the DV capsid protein, along with E and NS3, is an important target of the antiviral CTL response to DV infection in some individuals.

MATERIALS AND METHODS

Viruses. D1V (Hawaii strain) and D2V (New Guinea-C strain) were provided by Walter E. Brandt, Walter Reed Army Institute of Research. D3V (strain CH53489) was provided by Bruce L. Innis, then at the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, and D4V (Caribbean strain 814669) was provided by Jack McCown, Walter Reed Army Institute of Research. Yellow fever virus (YFV; 17D strain) was provided by Jacob J. Schlesinger, University of Rochester School of Medicine and Dentistry. West Nile virus (WNV; E101 strain) was provided by Margo Brinton, Georgia State University. The viruses were propagated in C6/36 mosquito cells to titers of 10^7 to 10^8 PFU/ml by plaque assay on CV-1 cell monolayers as previously described (22).

Preparation of flavivirus antigens. D1V, D2V, D3V, D4V, WNV, and YFV antigens were prepared from infected Vero cell monolayers as previously described (24). Briefly, Vero cell monolayers were infected at a multiplicity of infection of 1.0 PFU per cell and incubated at 37°C in minimal essential medium supplemented with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml and containing 2% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) until 50% of the cells displayed cytopathic effects. Cells were then harvested by scraping, washed, fixed in 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 1640. The suspension of fixed cells was then sonicated on ice in a sonic dismembrator (Fisher Chemical Co.) and centrifuged at $1,500 \times g$ for 10 min at 4°C. The supernatant was collected, aliquoted, and frozen at −70°C as viral antigen. Control antigen was prepared similarly from uninfected Vero cell monolayers.

Recombinant BV-derived DV antigens. *Spodoptera frugiperda* (Sf9) cells were infected with wild-type and recombinant baculoviruses (BV) and prepared as previously described (37). Sf9 cells infected with a recombinant BV containing a 4.0-kb cDNA sequence of D4V strain 814669 have been shown to express D4V C, premembrane (pre-M), E, NS1, and NS2a proteins (9). Recombinant BV containing shorter cDNA sequences for the D4V E, pre-M, and NS1 proteins were also used to infect Sf9 cells. These antigens were provided by C. J. Lai, National Institute of Allergy and Infectious Diseases.

Human PBMC. Peripheral blood specimens were obtained from the donor 6 months postvaccination with an experimental live-attenuated D4V vaccine, 314750 (16, 28). The vaccine virus was passaged 15 times in primary canine kidney cells and then four times in fetal rhesus lung cells. The donor received 4.8×10^5 PFU of virus in 0.5 ml subcutaneously. The donor did not have antibody to DV prior to vaccination. A very low titer antibody to YFV (1:10) was present in this donor prior to vaccination, probably as a result of a natural infection because this donor had no history of a prior YFV vaccination but had travelled in areas where yellow fever is endemic. Postvaccination, the donor developed very high titers of antibody to D4V: hemagglutination inhibition titer, ≥1:1,000; neutralization titer, ≥1:120 (15a). PBMC were purified by Ficoll-Hypaque density gradient centrifugation (3). Cells were resuspended at 10^7/ml in RPMI 1640 with 10% FBS (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use. The HLA type of this donor was A26, A28, B27, B39, Cw1, DR1, DR4, DPw4, DQ1, DQ3, and DR53. HLA DQ and DR were typed serologically at the HLA typing laboratory at the University of Massachusetts. HLA DP was typed by PCR by Elaine Reed at Columbia University.

Proliferative responses of PBMC. Proliferation assays of PBMC were performed as previously described (24). PBMC (2×10^5) were cultured with viral antigens or peptides at various dilutions in 0.2 ml of AIM-V medium (GIBCO) containing 10% heat-inactivated human AB serum (Hu ABS; Advanced Biotechnologies, Inc., Columbia, Md.) in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.) at 37°C for 7 days. Since the exact titers of the viral antigen preparations were not known, a range of dilutions from 1:40 to 1:640 was originally tested. The cells were pulsed with 1.25 μCi of tritiated thymidine ($[^3H]$TdR) for 8 to 14 h before harvest with a multiharvester (Titertek; Skatron Inc., Sterling, Va.). $[^3H]$TdR incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland).

Bulk culture of PBMC. PBMC were suspended at 5×10^6/ml in AIM-V medium containing 10% Hu ABS, 10% T-STIM culture supernatant (Collaborative Biomedical Products, Bedford, Mass.), penicillin-streptomycin, glutamine, and HEPES, with D4V antigen (D4 Ag) at a final concentration of 1:320, in 24-well cluster plates (Costar). On day 7 of culture, cells were either cloned by limiting dilution as described below or restimulated with 2×10^6 gamma-irradiated (3,000 rad) autologous PBMC in 1 ml of fresh medium containing 20% Hu ABS, 20% T-STIM, and D4 Ag at a final dilution of 1:80. Restimulated cells were assayed 7 days later for cytolytic activity.

CTL clones. DV specific CTL clones were established by using a limiting dilution technique as previously described (25). PBMC which had been stimulated in bulk culture for 7 days were collected and plated at a concentration of 1, 3, 10, or 30 cells per well in 96-well round-bottom microtiter plates in 150 μl of AIM-V medium containing 10% Hu ABS, 10% T-STIM, and D4 Ag at 1:320 and 10^5 gamma-irradiated autologous PBMC. On day 4, 50 μl of fresh medium with Hu ABS and T-STIM was added. On day 7, 100 μl of supernatant was removed, and fresh medium was added as described above along with 10^5 gamma-irradiated autologous PBMC and D4 Ag. Growing cells were expanded on days 14 and 21 into 48-well plates (Costar) and restimulated with 10^6 gamma-irradiated autologous PBMC in a final volume of 1 ml.

Establishment of lymphoblastoid cell lines. PBMC (2×10^6) were cultured in RPMI 1640 containing 20% FBS, penicillin, streptomycin, glutamine, and HEPES in the presence of 1:3 diluted Epstein-Barr virus from an infected marmoset cell line, B95-8 (American Type Culture Collection), in 24-well flat-bottom plates (Costar) (34). Cyclosporin was added at a final concentration of 1 μg/ml.

Persistent infection of BLCL with D2V. We established persistently infected cell lines by using D2V rather than D4V because of the difficulty of infecting cells with D4V in vitro. A total of 2×10^5 cells from Epstein-Barr virus-transformed B-lymphoblastoid cell lines (BLCL) from the donor were washed in minimal essential medium containing 2% FBS and incubated in 100 μl of minimal essential medium–2% FBS plus 100 μl of Raji cell-passaged D2V for 2 h at 37°C with frequent mixing. After infection, cells were transferred to a 24-well plate and 1 ml of RPMI 1640 containing 20% FBS was added. The percentage of infected cells was determined at various times by fluorescent antibody staining.

Cell surface antigen analysis. A total of 5×10^5 cells were washed twice in ice-cold PBS and stained with fluorescein isothiocyanate-conjugated anti-Leu2 (anti-CD8), anti-Leu3 (anti-CD4), and anti-Leu4 (anti-CD3) (Becton Dickinson Co., Mountain View, Calif.) antibodies as previously described (25). Control cells were labeled with conjugated normal mouse immunoglobulin G. Labeled cells were then washed three times in ice-cold PBS. Clones were then analyzed for CD4 or CD8 expression, using a fluorescence microscope.

Peptide synthesis. The original 15-mer peptides spanning the length of the D4V capsid protein were synthesized by using the RAMPS Multiple Peptide Synthesis System (New England Nuclear Products, Boston, Mass.) as previously reported (8, 36). Additional peptides were synthesized by using a Symphony Peptide Synthesizer (Rainin Instruments, Woburn, Mass.) at the University of Massachusetts Peptide Core Facility.

Preparation of target cells. Lymphoblastoid cells (5×10^5) were cultured for 16 to 20 h in RPMI 1640 containing 20% FBS and DV antigens diluted 1:80 or BV antigens diluted 1:100. The cells were then washed and labeled by incubation in 100 μl of RPMI 1640 containing 10% FBS and 0.25 mCi of ^{51}Cr (Dupont NEN, Boston, Mass.) for 60 min at 37°C. After labeling, cells were washed four times in RPMI 1640–10% FBS to remove unincorporated ^{51}Cr and then were counted and diluted to 10^4 cells per ml for use in a cytotoxicity assay. Control targets were prepared similarly but were not pulsed with viral antigens. We performed initial experiments in which uninfected Vero cell antigen was used to pulse negative control targets, and lysis was consistently the same as that of unpulsed targets.

Cytotoxicity assays. Assays were performed in 96-well round-bottom plates as previously reported (4). Effector cells in 100 μl of RPMI 1640 containing 10% FBS were added to 10^3 ^{51}Cr-labeled target cells in 100 μl at effector/target (E/T) ratios of 50:1 to 100:1 for bulk cultures and 3:1 to 30:1 for CTL clones. In CTL assays using synthetic peptides, peptides were added in 50 μl to 10^3 target cells in 100 μl and incubated for 20 min at 37°C, and 50 μl of effector cells was then added. Plates were incubated at 37°C for 4 to 6 h and then spun at $200 \times g$ for 5 min. Supernatant fluids were harvested, and ^{51}Cr content was measured in an automatic gamma counter (Packard, Sterling, Calif.). The percent specific chromium release was calculated from the following formula: [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100. The assays were done in triplicate, and results were calculated from the average of the triplicate wells. The standard error was less than 10% in all experiments. Experiments were performed at least twice.

RESULTS

Memory T-cell responses to stimulation with noninfectious flavivirus antigens. We first examined the proliferative responses of the PBMC from a donor who had received an experimental live-attenuated D4V vaccine 6 months earlier.

TABLE 1. Proliferation of PBMC from a D4V vaccine recipient to DV and flavivirus antigens in bulk culture[a]

Antigen	[^{3}H]TdR incorporation (cpm) at viral antigen dilution of:	
	1:160	1:320
D1V	1,316	1,705
D2V	12,633	8,445
D3V	2,461	4,912
D4V	10,707	69,243
WNV	3,260	5,201
YFV	65,598	25,976
Control Vero cell	1,530	1,674

[a] PBMC (2×10^5) were incubated for 6 days in the presence of serial dilutions of DV, WNV, or YFV antigens or the control (no antigen). Wells were pulsed with 1.25 μCi of [^{3}H]TdR for 8 h, and [^{3}H]TdR incorporation was measured. The mean value for the no-antigen control was 600 cpm.

PBMC were cultured with various concentrations of noninfectious flavivirus antigens, and proliferation was measured on day 7 by uptake of [^{3}H]TdR. This donor's PBMC exhibited a strong proliferative response to D4 Ag along with lesser, cross-reactive proliferation to D2 Ag (Table 1). Control antigen induced minimal proliferation. The brisk proliferative response to YFV antigen may represent the memory response of this donor, probably as a result of a prior subclinical natural infection. It has been previously shown that the T cells that respond to these noninfectious DV antigen preparations are predominantly CD4$^+$ T cells (24, 25, 39). Thus, these results suggest that the CD4$^+$ T-cell response induced in this donor by the D4V vaccination is D2V/D4V cross-reactive.

Recognition of capsid protein by CD4$^+$ CTL clones. CD4$^+$ CTL clones were established from the donor's PBMC by limiting dilution as described in Materials and Methods. CTL clones were selected for further study on the basis of the ability to lyse D4 Ag-pulsed BLCL targets in a ^{51}Cr release assay as previously described (4). All of the clones studied were shown to be CD4$^+$ CD8$^-$ by fluorescent antibody staining (data not shown). The D4V protein specificity of 15 clones was tested in CTL assays in which targets were pulsed with noninfectious antigens made from Sf9 cells infected with recombinant BV expressing various D4V proteins. Seven of the 15 clones recognized the D4V capsid protein (Table 2). Targets pulsed with antigen prepared from a recombinant BV expressing the D4V capsid protein in conjunction with the pre-M, E, NS1, and NS2a proteins were lysed by these clones. However, targets pulsed with wild-type BV antigen or BV antigens containing pre-M, E, or NS1 alone were not lysed. In other assays, targets infected with a recombinant vaccinia virus expressing the D4V NS1 and NS2a proteins were also not lysed by these clones (data not shown). These data indicate that the live-attenuated D4V vaccine elicited immune responses at least partially directed against the D4V capsid protein.

DV serotype reactivity of capsid-specific CD4$^+$ CTL clones. The capsid-specific CTL clones were tested in a ^{51}Cr release assay against BLCL targets pulsed with DV antigens of all four serotypes to determine their patterns of serotype reactivity. Table 3 shows that the majority of these clones were able to lyse both D2 and D4 Ag-pulsed targets to similar degrees, indicating that they are cross-reactive between these two serotypes of DV. In contrast, one C-specific CTL clone was found to lyse only D4 Ag-pulsed targets and thus is DV serotype specific. No significant lysis was observed for targets pulsed with control antigen or targets pulsed with WNV or YFV antigens (data not shown). These data suggest that the CD4$^+$ CTL response to the D4V capsid protein in this donor has both serotype-specific and serotype-cross-reactive components.

TABLE 2. Recognition of D4V capsid protein by DV-specific CD4$^+$ CTL clones

Clone	E/T ratio	% Specific ^{51}Cr release for[a]:					
		D4 Ag	Antigen from[b]:				
			Wild-type BV	BV expressing:			
				C-NS2a[c]	Pre-M	E	NS1
Expt 1							
7E4	17:1	60	0	32	0	0	1
6E2	20:1	59	1	30	4	9	4
8G9	20:1	58	1	26	2	3	3
Expt 2							
8H8	16:1	59	0	28	1	0	0
8G7	22:1	53	0	22	0	0	0
8G5	20:1	52	3	23	4	5	0
5C8	16:1	51	2	30	0	2	0

[a] Specific levels of lysis are underlined.
[b] Targets were pulsed with antigens made from BV-infected SF9 cells as described in Materials and Methods.
[c] A segment of the D4V genome encoding C, pre-M, E, NS1, and NS2a (9).

Lysis of D2V-infected cells. Autologous BLCL were persistently infected with D2V and were used as targets in a CTL assay as described in Materials and Methods. Table 4 shows that the six D2V/D4V-crossreactive clones were able to recognize and lyse the D2V-infected targets whereas, as expected, the D4V-specific clone did not lyse these targets.

Capsid protein epitope mapping. We synthesized 15-mer peptides that spanned the majority of the D4V capsid protein. These peptides were tested for recognition by the capsid-specific CTL clones. As shown in Table 5, all of the D2V/D4V-crossreactive clones recognized only a peptide from aa 81 to 95 (LIGFRKEIGRMLNIL; D4V-C 81-95), while the D4V-specific clone recognized a separate 15-mer, from aa 41 to 55 (KGPLRMVLAFITFLR; D4V-C 41-55). At higher peptide concentrations, such as 25 μg/ml, targets pulsed with peptide and targets exposed to viral antigen were lysed at similar levels, although high peptide concentrations were sometimes moderately toxic to the target cells. At a peptide concentration of 2.5 μg/ml, lysis of peptide-pulsed targets was less than that of antigen-pulsed targets, but toxicity was also minimized.

Next, sequential truncations of the original 15-mer peptides

TABLE 3. DV serotype specificities of CD4$^+$ CD8$^-$ CTL clones[a]

Clone	% Specific ^{51}Cr release for[b]:			
	D1 Ag	D2 Ag	D3 Ag	D4 Ag
Expt 1				
7E4	3	71	0	62
8H8	10	50	3	57
8G9	0	78	0	66
8G7	0	50	2	58
8G5	0	46	0	38
Expt 2, 6E2	2	42	0	50
Expt 3, 5C8	5	0	2	40

[a] E/T ratios of 15:1 to 20:1 were used.
[b] Calculated as described in Materials and Methods. Specific levels of lysis are underlined. In each case, no lysis was found in the absence of antigen.

TABLE 4. Lysis of D2V-infected targets by $CD4^+$ CTL clones

Clone	E/T ratio	% Specific ^{51}Cr release for[a]:		
		No antigen	D4 Ag	D2 virus[b]
D2V/D4V cross-reactive				
6E2	8:1	0	17	15
7E4	11:1	0	64	54
8G9	9:1	0	28	32
8G7	12:1	0	58	53
8G5	5:1	0	24	42
8H8	4:1	3	47	48
5C8 (D4V specific)	8:1	0	23	0

[a] Specific levels of lysis are underlined.
[b] D2V-infected targets were persistently infected autologous BLCL as described in Materials and Methods. At the time of this experiment, 85% of the cells were infected as determined by fluorescent antibody staining.

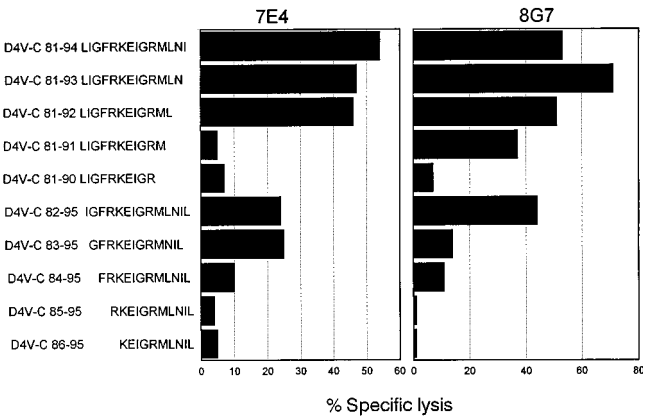

FIG. 1. Localization of epitopes on D4V capsid protein recognized by two representative $CD4^+$ CTL clones specific for D4V-C 81-95. Target cells were autologous BLCL incubated with CTL clones and the indicated peptide (25 μg/ml) for 5 h. E/T ratios were 4:1 for 7E4 and 7:1 for 8G7.

from both the C and N termini were synthesized and tested in similar assays. The results of representative experiments for two CTL clones that were originally found to recognize peptide 81-95 are shown in Fig. 1. By comparing the percent specific lysis for each of the truncations, an approximation of the minimum epitope needed for recognition by the clones can be estimated.

The results of these experiments were next confirmed by synthesizing peptides corresponding to the estimated minimum epitope and analyzing their recognition. Results for the six clones recognizing the original 15-mer peptide 81-95 are shown in Table 6. The results indicate that clones 7E4, 6E2, and 8G9 all recognize a minimum epitope composed of aa 84 to 92, while 8G5 and 8H8 recognize aa 84 to 91 and 8G7 recognizes aa 83 to 90. Comparison of the results of Fig. 1 and Table 6 shows that longer peptides containing the entire minimum epitope for certain clones are often less well recognized than the minimum epitope itself. This may be due to either decreased binding of the longer peptides to major histocompatibility complex (MHC) molecules or decreased recognition of the peptide-MHC complex by the T-cell receptor as a result of steric inhibition. Peptide 83-92 is entirely conserved between D2V and D4V, which explains the cross-reactivity of these clones between the two viral serotypes (17, 38).

The minimum epitope for the D4V-specific clone, 5C8, was found in a similar manner to extend from aa 47 to 55 (Table 7).

HLA restriction of capsid-specific $CD4^+$ CTL clones. The HLA class II alleles of this donor are DR1, DR4, DP4, DQw1, DQw3, and DRw53. To determine the HLA restriction of the $CD4^+$ CTL clones, CTL assays were performed with a panel of partially HLA-matched allogeneic BLCL pulsed with D4 Ag or the peptide epitope as targets. Nonpulsed autologous BLCL were used as a negative control. In some experiments, nonpulsed allogeneic targets were used to show that lysis was not

TABLE 5. Recognition of two distinct epitopes on the D4V capsid protein by $CD4^+$ CTL clones[a]

Autologous LCL treated with[b]:	% Specific ^{51}Cr release[c]						
	D2V/D4V cross-reactive						D4V specific, expt 4
	Expt 1			Expt 2	Expt 3		
	8G9	6E2	8G7	7E4	8H8	8G5	5C8
No antigen	0	0	0	0	3	0	0
D4 Ag	45	48	28	46	47	24	73
D4V-C 2-15	0	0	0	0	0	0	4
D4V-C 21-35	0	0	0	0	0	0	0
D4V-C 31-45	0	0	0	0	0	0	4
D4V-C 36-50	6	5	0	0	0	0	—[d]
D4V-C 41-55	0	0	0	0	0	0	35
D4V-C 56-70	3	0	0	0	0	0	0
D4V-C 61-75	0	0	0	0	0	0	0
D4V-C 81-95	27	33	23	18	23	13	0
D4V-C 86-100	0	0	0	0	0	0	0
D4V-C 96-110	0	0	0	0	0	0	8

[a] E/T ratios used were 5:1 for 8G9, 6E2, and 8G7, 11:1 for 7E4, 5:1 for 8G5, 4:1 for 8H8, and 7:1 for 5C8.
[b] Peptides were present at a final concentration of 2.5 μg/ml for the duration of the assay.
[c] Specific levels of lysis are underlined.
[d] —, not determined.

TABLE 6. Variation in minimum epitopes recognized by $CD4^+$ CTL clones

Peptide	Amino acid sequence	Concn (μg/ml)	% Specific ^{51}Cr release[a]					
			7E4 (10:1[b])	8G5 (6:1)	8H8 (10:1)	8G9 (16:1)	6E2 (15:1)	8G7 (12:1)
D4V-C 81-89	LIGFRKEIG	2.5	0	1	1	0	0	2
		0.25	0	1	0	0	0	0
		0.025	0	1	—[c]	—	0	—
D4V-C 83-90	GFRKEIGR	2.5	64	0	5	0	0	54
		0.25	40	0	0	0	0	24
		0.025	5	0	—	—	0	—
D4V-C 84-92	FRKEIGRML	2.5	82	27	33	46	63	17
		0.25	78	21	9	0	34	0
		0.025	41	5	—	—	3	—
D4V-C 84-91	FRKEIGRM	2.5	70	36	49	13	1	10
		0.25	56	24	36	0	0	0
		0.025	11	7	—	—	0	—
D4V-C 85-92	RKEIGRML	2.5	10	4	0	0	5	0
		0.25	2	0	0	0	2	0
		0.025	6	0	—	—	2	—
D4V-C 85-91	RKEIGRM	2.5	6	0	0	0	0	0
		0.25	2	0	0	0	0	0
		0.025	0	0	—	—	0	—

[a] Each clone represents a separate experiment. Peptides were present for the duration of the experiment. Specific levels of lysis are underlined.
[b] E/T ratio.
[c] —, not determined.

TABLE 7. Recognition of aa 47 to 55 on the D4V capsid protein by $CD4^+$ CTL clone 5C8

Peptide	Amino acid sequence	Concn (μg/ml)	% Specific ^{51}Cr release[a]
Expt 1			
D4V-C 43-55	PLRMVLAFITFLR	2.5	80
		0.25	80
		0.025	40
D4V-C 46-55	MVLAFITFLR	2.5	61
		0.25	46
		0.025	1
D4V-C 45-54	RMVLAFITFL	2.5	14
		0.25	0
		0.025	0
D4V-C 46-54	MVLAFITFL	2.5	17
		0.25	5
		0.025	0
Expt 2			
D4V-C 47-55	VLAFITFLR	2.5	79
		0.25	20
		0.025	1
D4V-C 48-55	LAFITFLR	2.5	22
		0.25	0
		0.025	0

[a] E/T ratios were 14:1 in experiment 1 and 16:1 in experiment 2. Specific levels of lysis are underlined.

TABLE 8. MHC class II restriction of $CD4^+$ CTL clones

Target	HLA class II type[a]			% Specific ^{51}Cr release[b]						
	DR	DP	DQ	6E2	5C8	7E4	8H8	8G5	8G9	8G7
Expt 1										
Autologous	1,4	w4	1,3	68	27	39				
9011	2	w2,w4	1	48	34	33				
9052	7	w4	9	29	34	17				
CP	5,7	w4	2,3	0	26	0				
CB	7	w4	2	35	20	19				
Expt 2										
Autologous	1,4	w4	1,3			53			18	26
FS	1,6	w3,w4	1			52			23	32
3104	1	—	1			61			13	31
3099	1	—	1			38			13	20
9052	7	w4	9			15			1	0
9011	2	w2,w4	1			38			4	5
3107	2	w4	1			31			0	0
Expt 3										
Autologous	1,4	w4	1,3				66	46	75	60
9004	1	w4	1						88	49
3099	1	—	1				38	35	68	50
FS	1,6	w3,w4	1				59	24	33	28
9052	7	w4	9				0	0	0	2
JK	2	w2	1				1	3	0	0
9087	3	w3,w4	2				0	1	0	0

[a] HLA loci known to match donor's HLA are underlined. —, locus for which HLA haplotype is unknown.

[b] In experiment 1, E/T ratios were 10:1 for 6E2 and 8H8, 12:1 for 7E4, 7:1 for 8G7, 8:1 for 8G5, and 14:1 for 5C8. Targets for 5C8 were pulsed with D4V-C 41-55 at 1 μg/ml, and targets for the remaining clones were pulsed with D4V-C 81-95 at 7.6 μg/ml. In experiment 2, E/T ratios were 20:1 for 6E2, 8G9, and 8G7, 19:1 for 7E4, 18:1 for 8H8, and 12:1 for 8G5. Targets for 8G5 were pulsed with D4V-C 81-95 at 7.6 μg/ml, and targets for the remaining clones were pulsed overnight with D4 Ag at 1:70. In experiment 3, E/T ratios were 5:1 for 7E4, 2:1 for 8G9, and 9:1 for 8G7. Targets were pulsed with D4V-C 81-95 at 7.6 μg/ml. In experiment 4, E/T ratios were 12:1 for 8G7, 14:1 for 8G9, and 6:1 for 8G5. Targets were pulsed overnight with D4 Ag at 1:80. Specific levels of lysis are underlined.

due to allorecognition (data not shown). The results in Table 8 show that clones 7E4, 6E2, and 5C8 are able to lyse autologous targets that share only DPw4, for example, 9052 and CB, indicating that these clones are restricted by HLA DPw4. The inability of these clones to lyse the CP target, which also expresses DPw4, is likely due to variations present in the DPw4 allele of this target that affect peptide presentation. The results of this assay indicate that targets 3099 and 3104, for which the DP type is unknown, likely bear HLA DPw4. Clones 8H8, 8G9, and 8G7 were unable to lyse target cells which shared DR4, DQ1, and DQ3 (data not shown), but these three clones and 8G5 are able to lyse pulsed target cells that express DR1 and not targets that share only DP4 (9052 and 9087), indicating that these clones are DR1 restricted.

Recognition of capsid epitope by T cells in short-term bulk cultures. The $CD4^+$ CTL response in this donor seemed to be primarily directed against aa 84 to 92 of the capsid protein at the clonal level, and therefore we determined whether this peptide epitope was able to induce T-cell proliferation in a short-term bulk culture. Figure 2 shows that peptides containing the epitope from aa 84 to 92, which were recognized by the $CD4^+$ CTL clones, induced proliferation in a 7-day bulk culture, while peptides that did not contain the epitope did not. For peptide 84-92, the proliferation is higher at 2.5 than at 25 μM, probably because of toxicity at the higher concentration. CTL killing was also examined at the bulk culture level. CTL generated in a 14-day bulk culture with D4 Ag recognized and lysed targets pulsed with peptides containing the minimum epitope 84-92, and these CTL did not kill targets pulsed with peptides not recognized by the clones (Fig. 3). These results indicate that the recognition of an epitope from aa 84 to 92 on the D4V capsid protein is present in early bulk cultures as well as at the clonal level.

DISCUSSION

In this study, we analyzed the $CD4^+$ T-cell responses of a donor who had received an experimental live-attenuated D4V vaccine. In bulk culture, PBMC from this donor exhibited a brisk proliferative response to noninfectious D4 Ag, with lesser, cross-reactive proliferation observed to D2 Ag. The $CD4^+$ CTL clones which we established recognize D4V capsid protein. To our knowledge, this is the first report of human CTL epitopes on the DV capsid protein. Previously, human

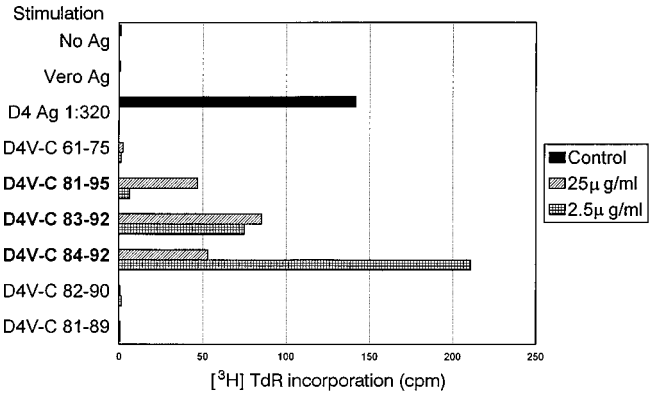

FIG. 2. Proliferation of PBMC in short-term culture with peptides containing the D4V-C epitope. PBMC (2×10^5 per well) were stimulated for 7 days in the presence of D4 Ag, control antigen, or peptides at 25 or 2.5 μg/ml. Peptides containing the epitope are listed on the *y* axis in boldface.

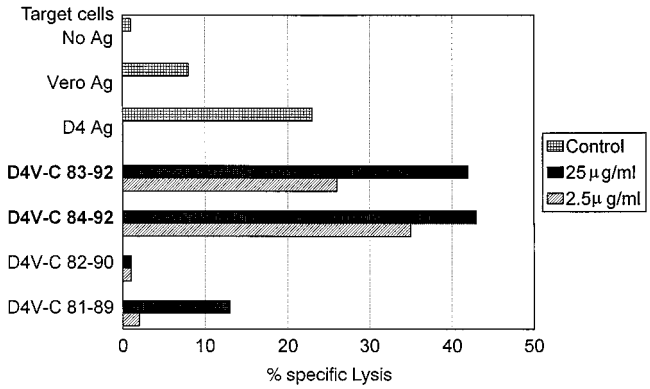

FIG. 3. Lysis of targets pulsed with peptides encompassing the D4V-C epitope by PBMC from a short-term bulk culture. PBMC were stimulated for 14 days in the presence of noninfectious D4 Ag as described in Materials and Methods. Target cells were autologous BLCL pulsed with D4 Ag or the indicated peptide in a 4.5-h assay. The E/T ratio was approximately 50:1. Peptides containing the epitope are indicated in boldface.

$CD8^+$ and $CD4^+$ CTL epitopes have been demonstrated on the E (4, 27) and the NS3 (20, 26) proteins.

The capsid-specific CTL clones were found to be composed of two subgroups with regard to DV serotype specificity. One of the clones was D4V specific and recognized a 9-mer minimum epitope from aa 47 to 55. The six remaining capsid-specific clones were D2V/D4V cross-reactive and recognized slight variations in a minimum epitope located between aa 83 and 92. The serotype reactivity of each clone is explained by the peptide sequences of the minimum epitopes (Table 9). The sequence of the D4V capsid peptide from aa 47 to 55 is different from those of the peptides from the other three serotypes of the virus, while the sequences of D2V and D4V aa 83 to 92 are identical but different from those of D1V and D3V at three positions (17, 29, 31, 38). These results are consistent with those found for other DV-immune donors in that both serotype-specific and serotype-cross-reactive $CD4^+$ CTL have also been identified in these individuals (11, 20, 25).

It is interesting that the cross-reactive capsid protein epitope recognized by these $CD4^+$ CTL clones seems to be presented in the context of two different HLA class II molecules, as some of the clones are restricted by HLA DR1 and others are restricted by DP4. Such promiscuity in class II MHC binding has been observed previously with other peptides (10, 18, 32) and can usually be explained by differences in the exact minimum epitope bound to the individual alleles. The same may be true in this case because the capsid-specific clones exhibit some variability in the minimum peptide that they can recognize and in the lowest peptide concentration at which they are able to recognize that epitope. The slight variations in the recognition patterns of these clones might also be attributable to the different T-cell receptors that they utilize. Analysis of the Vα and Vβ expression of these clones has shown that there is heterogeneity in the usage of both of these receptor subunits (30a).

It is sometimes argued that long-term in vitro-generated CTL clones may be biased toward recognition of an epitope that is not necessarily relevant in vivo. This report presents evidence suggesting this is not necessarily the case. First, the six D2/D4-cross-reactive CTL clones lysed cells that were actively infected with D2V, indicating that the relevant epitope from the DV capsid protein is actually processed and presented in association with MHC class II molecules on DV-infected cells. Secondly, short-term bulk culture assays indicate that the recognition of the C protein epitope is not merely a clonal phenomenon. Bulk culture experiments using the capsid peptide epitope from aa 84 to 92 demonstrated that this epitope is capable of inducing proliferation of the donor's PBMC in a 7-day culture. Also, effector cells in short-term PBMC cultures stimulated with noninfectious D4 Ag were able to specifically lyse targets pulsed with the same capsid epitope. These experiments indicate that this capsid protein epitope is likely to be important in the in vivo immune response of this donor to the D4V vaccine as well as being recognized at the clonal level. Future experiments will look more directly at the potential dominance of this epitope in the donor's memory response to DV by examining the precursor frequency of capsid-specific T cells compared with that of DV-specific T cells.

The role of $CD4^+$ T cells in DV infection is still poorly understood. Levels of soluble CD4 and other markers of T-cell activation were shown to be significantly higher in the sera of Thai children with DHF than in children with simple dengue fever (DF) or healthy children (23), suggesting that there are more $CD4^+$ T cells activated during DHF than during DF. It is reasonable to hypothesize that high levels of $CD4^+$ as well as $CD8^+$ T-cell activation may be related to the pathogenesis of this disease. This study and others (11, 20, 25) have shown that cross-reactive memory $CD4^+$ CTL are generated after DV infection, and stimulation of these cross-reactive memory T cells during a secondary infection with a heterologous serotype of DV may result in the high levels of T-cell activation. Activated T cells will secrete lymphokines which may contribute to the pathogenesis of DHF and may lyse virus-infected cells, causing the release of other vasoactive mediators. Further studies are necessary to delineate the connection between high levels of T-cell activation and the pathogenesis of DHF. These studies will include an in vivo analysis of cytokine levels in subjects with DHF and in those with DF or healthy subjects, as well as an analysis of protein recognition by DV-specific T cells activated in vivo during DV infection.

TABLE 9. Amino acid sequences[a] of D1V to D4V at epitopes recognized by capsid-specific $CD4^+$ CTL clones

Epitope	Sequence[b]									
1 (aa 47–55)										
D4V	V	L	A	F	I	T	F	L	R	
D1V		M				A				
D2V	F	M		L	V	A				
D3V		M				A				
2 (aa 83–92)										
D4V	G	F	R	K	E	I	G	R	M	L
D1V			K				S	N		
D2V										
D3V			K				S	N		

[a] From references 16, 27, 29, and 36.
[b] Blank cells are the same as the D4V sequence.

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