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A Single Nine-amino acid Peptide Induces Virus-specific, CD8+ Human Cytotoxic T Lymphocyte Clones of Heterogeneous Serotype Specificities

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Summary

It is generally accepted that virus-specific CD8+ cytotoxic T lymphocytes (CTLs) recognize nine-amino acid peptides in conjunction with HLA class I molecules. We recently reported that dengue virus-specific CD8+ CTLs of two different serotype specificities, which were established by stimulation with dengue virus, recognize a single nine-amino acid peptide of the nonstructural protein NS3 of dengue virus type 4 (D4V) in an HLA-B35-restricted fashion. To further analyze the relationships between the serotype specificities of T cells and the amino acid sequence of the recognized peptides, we examined the ability of this viral peptide D4.NS3.500-508 (TPEGIIPTL) to stimulate T lymphocytes of an HLA-B35-positive, dengue virus type 4-immune donor. Peptide stimulation of the PBMC generated dengue virus-specific, HLA-B35-restricted CD8+ CTL clones. These clones lysed dengue virus-infected autologous cells, as well as autologous target cells pulsed with this peptide. Four patterns of dengue virus serotype specificities were demonstrated on target cells infected with dengue-vaccinia recombinant viruses or pulsed with synthetic peptides corresponding to amino acid sequences of four dengue virus serotypes. Two serotype-specific clones recognized only D4V. Three dengue virus subcomplex-specific clones recognized D1V, D3V, and D4V, and one subcomplex-specific clone recognized D2V and D4V. Three dengue virus serotype-cross-reactive clones recognized D1V-D4V. Thus, a single nine-amino acid peptide induces proliferation of a heterogeneous panel of dengue virus-specific CD8+ CTL clones that are all restricted by HLA-B35 but have a variety of serotype specificities. Peptides that contain a single amino acid substitution at each position of D4.NS3.500-508 were recognized differently by the T cell clones. These results indicate that a single epitope can be recognized by multiple CD8+ CTLs that have a variety of serotype specificities, but the manner of recognition by these multiple CTLs is heterogeneous.

Human T lymphocytes recognize antigenic peptides that are bound to HLA (1, 2). TCRs consist of a heterodimer of α and β chains expressed on the cell surface and are responsible for recognition of the peptide-HLA complexes (3, 4). The specificity of T lymphocytes is, therefore, determined by the interaction of TCRs with peptide-HLA complexes. It is generally accepted that the V(D)J junction (complementarity determining region [CDR][1] 3 portion) of TCR is mainly responsible for recognition of peptide, and that the CDR1 and CDR2 portions recognize HLA (5); however, how the TCR interacts with peptide and HLA is not completely understood (6).

Virus-specific CTLs play an important role in recovery from infection (7, 8) and in immunopathological mechanisms that may occur in viral infections (9-11). Virus-specific CD8+ CTLs are heterogeneous in their serotype specificities: serotype-specific CTLs that recognize one serotype of the virus and serotype-cross-reactive CTLs that recognize more than one serotype (12). The peptides that bind to HLA class I molecules and are recognized by HLA class I-restricted CD8+ CD4- CTLs are usually nine-amino acid peptides (13).

[1] *Abbreviations used in this paper:* CD, complimentarity determining region; DHF, dengue hemorrhagic fever; D1V-D4V, dengue virus types 1-4; LCL, B lymphoblastoid cell lines; TCGF, T cell growth factor; VV, vaccinia virus; VV-DV, vaccinia dengue recombinant virus.

Serotype specificity and serotype cross-reactivity of virus-specific CD8+ CTLs are believed to be determined by the degree of conservation of the amino acid sequences among serotypes. Thus, it is likely that epitopes that are not conserved among serotypes of certain viruses are recognized by serotype-specific CTLs, while epitopes that are conserved among serotypes are recognized by serotype–cross-reactive CTLs. Although this may be generally the case, we have recently described dengue virus–specific CTL clones that have different serotype specificities: dengue virus type 4 (D4V) serotype–specific and D4V–D2V cross-reactive, but recognizing the same nine–amino acid peptide in an HLA-B35–restricted fashion (14). This result suggests that serotype specificity and cross-reactivity may be determined, not only by the degree of conservation in the amino acid sequences of the epitopes, but also by the T cell itself via its TCR. T cells that possess a very specific TCR are serotype specific, while T cells that possess a less specific TCR are serotype cross-reactive, even though these two groups of T cells recognize the same epitope.

In this paper, we further elucidate the relationship between the amino acid sequences of a peptide and the serotype specificities of the T cells that recognize the peptide. After stimulation of PBMC with a single nine–amino acid peptide, we established virus-specific CTL clones that recognize the stimulating peptide with the same HLA restriction but have heterogeneous serotype specificities. These results suggest that T cell serotype specificities and cross-reactivities may not be solely determined by the degree of amino acid conservations of the epitopes among serotypes.

Materials and Methods

Viruses. D2V (New Guinea C strain) and D4V, (814669 strain), were used in this study. These viruses were propagated in C6/36 mosquito cells as previously described (15). Briefly, C6/36 cell monolayers were infected at a multiplicity of infection of 0.1 PFU per cell and were incubated for 7 d at 28°C in MEM containing 2% FCS (GIBCO BRL, Gaithersburg; MD) and 0.8% BSA, and the supernatant was collected and stored at −70°C. Viral titers of the supernatants were 10^7–10^8 PFU/ml by plaque assay on CV-1 cell monolayers (16).

The NS3 gene of D2V (New Guinea C strain) and D3V (H-87 strain) were amplified from appropriate genomic RNAs using reverse transcription PCR with primers that contained BamH1 and HindIII restriction sites. The resulting PCR product was ligated into the vaccinia transfer plasmid, pKG19, which contains both BamH1 and HindIII restriction sites in its cloning cassette. Transfection of pGK19 vectors into vaccinia virus (VV) strain WR-infected CV-1 cells results in recombination at the viral thymidine kinase gene locus. VV recombinants that contained the dengue NS3 gene were isolated after growth in BudR, confirmed by PCR, plaque purified three times, and grown to titers of 0.5–5 × 10^8 PFU/ml on CV-1 cells. The presence of the NS3 gene in VV-infected CV-1 cells was demonstrated by SDS-PAGE. An additional VV, which contains the NS3 gene of D4V (814669 strain), was previously provided by Dr. C.-J. Lai (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (17, 18).

Human PBMC. PBMC from a healthy adult who had been immunized with a live attenuated experimental D4V vaccine 1 yr earlier were collected by leukophoresis and separated by Ficoll-Hypaque density gradient centrifugation (19). PBMC were resuspended in RPMI containing 20% FCS and 10% DMSO, and frozen in a programmable cryopreservation chamber (Cryo-Med, Mt. Clemens, MI). The HLA type of this donor was A2, A23; B35; Cw4; DR7, DR53; DQ2; DPw4.

Preparation of Dengue Virus Type 4 Antigen. D4V antigen was prepared from infected Vero cell monolayers as previously described (20). Briefly, Vero cell monolayers were infected at a multiplicity of infection of 1.0 PFU per cell and incubated at 37°C in MEM containing 2% FCS until 50% of the cells displayed cytopathic effects. Cells were then harvested by scraping, washed, fixed in 0.025% glutaraldehyde in 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 using a sonic dismembrator (Fisher Chemical Co., Pittsburgh, PA) and centrifuged at 1,500 *g* for 10 min at 4°C. The supernatant was collected, aliquoted, and frozen at −70°C. Control antigen was prepared similarly from uninfected Vero cell monolayers.

Peptide Synthesis. Peptides were synthesized using the RAMPS Multiple Peptide Synthesis System (New England Nuclear Products, Boston, MA), as previously reported (21–23). Some peptides were synthesized using the Symphony Peptide Synthesizer (Rainin Instruments, Woburn, MA) at the University of Massachusetts Peptide Core Facility. Analyses to verify the amino acid composition of the peptides and purification of the peptide D4.NS3.500-508 using HPLC were performed by Dr. Robert Carraway at the Peptide Core Facility.

Flow Cytometry Peptide Binding Assay. A peptide binding assay using RMA-S-B*3501 cells was performed as previously described (24). Briefly, RMA-S-B*3501 cells were cultured at 26°C for 18–24 h. Cells (6 × 10^5 in 50 μl of PBS supplemented with 20% FCS) were incubated at 26°C for 1 h with 50 μl of different concentrations of peptide solution in 96-well culture plates followed by incubation at 37°C for 3 h. After washing with PBS containing 20% FCS, the cells were stained with mAb to HLA-Bw6, SFR8-B6 (25), and FITC-conjugated IgG of sheep anti–mouse Ig antibodies (Silenus Laboratories, Hawthorn, Australia). Fluorescence intensity was measured using a FACScan®.

Cell Surface Antigen Analysis. 5 × 10^5 cells were washed twice in ice-cold PBS and stained with FITC-conjugated anti-Leu2 (anti-CD8), anti-Leu3 (anti-CD4), or anti-Leu4 (anti-CD3) as previously described (26). Control cells were labeled with FITC-conjugated normal mouse IgG. Labeled cells were then washed three times in ice-cold PBS. The surface fluorescence was quantitated by FACS® analysis (FACS® 440; Becton Dickinson & Co., Mountain View, CA), or the cells were analyzed for CD4 or CD8 expression with a fluorescence microscope.

Stimulation of D4V-immune PBMC with D4V. PBMC were suspended at 5 × 10^6 cells/ml in AIM-V medium containing 10% heat-inactivated human AB serum (Advanced Biotechnologies, Inc., Columbia MD). 5 × 10^6 cells in 1 ml were added to 1.0 ml of D4V in 24-well cluster plates (Costar Corp., Cambridge, MA), as previously described (27). Viral peptide–specific cytolytic activity was assayed using some of these cells after 7 d of culture at 37°C, 1.0 ml of D4V was then added to the rest of the cells and cytolytic activity was assayed again on day 14 at 37°C.

Proliferative Response of PBMC to Viral Peptides. PBMC (1.5 × 10^5 to 2.5 × 10^5) were cultured with synthetic viral peptides and viral antigen at various dilutions in 0.2 ml of AIM-V medium (GIBCO BRL) containing 10% human AB serum in 96-well round-bottom microtiter plates (Costar, Corp.) at 37°C for 6 d. The cells

were pulsed with 1.5 μCi of [^{3}H]thymidine for 10 h before harvest with a multiharvester (Titertek; Skatron Inc. Sterling, VA). [^{3}H]Thymidine incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland).

Establishment of Dengue Virus-specific T Cell Clones by Limiting Dilution. PBMC (6×10^6) were γ-irradiated (3,000 rads) and incubated for 2 h with peptide D4.NS3.500-508 at 1 μg/ml in 0.7 ml of RPMI containing 10% FCS. Cells were then washed five times in RPMI containing 10% FCS, centrifuged together with 4×10^6 autologous PBMC, and cultured in 48-well plates (Costar Corp.) for 7 d in 1 ml of AIM-V medium supplemented with 10% human AB serum. T cell growth factor (T-Stim; Collaborative Research, Inc., Bedford, MA) was added to the final concentration of 10% on day 3. On day 7, 2×10^6 γ- irradiated autologous PBMC were pulsed with peptide D4.NS3.500-508 at 1 μg/ml for 2 h and then washed five times. These γ-irradiated, peptide-pulsed PBMC were mixed and centrifuged together with growing cells and resuspended in 1ml of AIM-V medium containing 10% human AB serum and 10% T cell growth factor (TCGF) in 48-well plates. The growing cells were restimulated again on days 21 and 35. Half of the media in the wells was replaced every 3 d with AIM-V, 15% T cell growth factor and 15% human AB serum. 1 wk after the last restimulation on day 35, cells were seeded into V-bottom plates (Costar Corp.) at concentrations of 100, 10, 3, and 1 cells per well with 10^5 γ-irradiated autologous PBMC in 0.2 ml of AIM-V containing 10% human AB serum, 20 U/ml human rIL-2 (Collaborative Research, Inc., Bedford, MA) and 0.1 μg/ml of mAb to CD3 (12F6; kindly provided by Dr. Johnson Wong, Massachusetts General Hospital, Boston, MA). Every 3 d, 0.1 ml of supernatant was removed from each well and replaced by AIM-V containing 10% human AB serum and 20 U/ml IL-2. 2 wk after seeding, half of the cells in each well were examined for cytotoxicity against autologous B lymphoblastoid cell lines (LCL) infected with rVV containing the D4V NS3 gene (VV [D4:NS3]) or with control VV.

In one limiting dilution experiment, 33 out of 672 wells demonstrated cytotoxic activity that was greater than spontaneous release +2.56 × SEM. These cells were transferred to 48-well plates, and were further expanded. The percentages of the wells that contained the growing cells with specific cytotoxic activity were 4.9% with 1 cell per well, 4.2% with 3 cells per well, 8.3% with 10 cells per well, and 43% with 100 cells per well. The nine CTL clones used in the experiments were selected based on the specificity and growth. Clones 2, 9, 12, and 14 were established from the wells that contained 1 cell per well, clones 20, 21, and 22 were established from the wells that contained 3 cells per well, and clone 26 was established from the well that contained 10 cells per well.

Preparation of Target Cells. Autologous LCL were derived through transformation of peripheral blood lymphocytes with EBV as previously described (20) and were maintained in RPMI containing 10% FCS at 37°C. Culture fluid from the EBV transformed marmoset cell line, B95-8, was used as a source of EBV. Vaccinia-dengue recombinant virus (VV-DV)–infected target cells were prepared by infecting 10^6 LCL with VV-DV at a multiplicity of infection of ~20 PFU per cell for 2 h. After being washed twice, LCL were cultured in RPMI containing 10% FCS at 37°C for 16–20 h and were used as target cells.

D2V-infected LCL were established by infection of 4×10^5 LCL with 0.25 ml of an undiluted supernatant from D2V-infected Raji cells and were maintained in cultures for 2–6 wk before use as CTL targets. 50–70% of the D2V-infected LCL contained dengue virus antigen as detected by immunofluorescence using hyperimmune anti-D2V mouse ascites fluid 2 and 4 wk after infection.

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, MA) for 45–60 min in 0.1 ml 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.

^{51}Cr Release Cytotoxicity Assay. Cytotoxicity assays were performed in 96-well round-bottom plates as previously reported (22, 26). Effector cells in 0.1 ml of RPMI containing 10% FCS were added to 1×10^3 ^{51}Cr-labeled target cells in 0.1 ml at E/T ratios of 50–100:1 for bulk cultures and 5–10:1 for CTL clones. In cytotoxicity assays using synthetic peptides, 0.05 ml of synthetic peptides was added to 10^3 target cells in 0.1 ml, incubated at 37°C for 30 min, and 0.05 ml of effector cells were then added. All assays were performed in triplicate. After centrifugation at 200 *g* for 5 min, plates were incubated at 37°C for 4–5 h. Supernatant fluids were harvested and the ^{51}Cr content was measured in a gamma counter (Packard Instruments, Sterling, CA). The percent of specific release was calculated using the formula:

$$\frac{[(\text{cpm experimental release}) - (\text{cpm spontaneous release})] \times 100}{[(\text{cpm maximal release}) - (\text{cpm spontaneous release})]}.$$

Results

Proliferative Responses of Donor 1 PBMC to the Synthetic Viral Peptide D4.NS3.500-508 and Recognition of the Peptide by CTLs Generated in Bulk Culture. We have previously reported that the epitopes that are recognized by dengue virus–specific, HLA-B35-restricted CD8$^+$ CTL clones established by stimulation with D4V are located within amino acids 453–618 of NS3 (14). The motif for HLA-B35–binding peptides has been reported to be nine–amino acids peptides that contain a proline at the second position (28). Eight nine-amino acid peptides were prepared based on the HLA B35-binding motif from the amino acid sequence located between amino acids 453 and 618 of D4V NS3.

PBMC from this D4V-immune, HLA-B35–positive donor were cultured with each of these peptides and T cell proliferation was examined. PBMC proliferated when cultured with peptide D4.NS3.500-508 (TPEGIIPTL), but did not proliferate when cultured with the other peptides (Table 1). D4.NS3.500-508 did not induce proliferation of PBMC from a dengue virus–non immune, HLA-B35–positive donor or cord blood lymphocytes (data not presented).

We then examined whether D4.NS3.500-508 is recognized by CTLs generated by stimulation with DV4 in bulk culture (Table 2). PBMC from the D4V-immune donor were cultured with D4V for 7 d (Experiment 1), and further cultured with D4V for seven more days in the presence of 10% TCGF (Experiment 2). Lysis of autologous LCL that had been pulsed with each of the eight peptides by CTL generated in bulk cultures was examined. CTLs generated by stimulation with D4V in bulk cultures lysed target cells pulsed with D4.NS3.500-508, but did not lyse target cells pulsed with the other peptides. These results suggest that D4.NS3.500-508 is the only HLA-B35–restricted epitope recognized by D4V-specific CTLs between amino acids 453 and 618 on NS3.

We quantified the binding of these peptides to HLA-B*3501 molecules by flow cytometry analysis of RMA-S cells transfected with the HLA-B*3501 gene as described in Materials

Table 1. *Stimulation of PBMC from D4V-immune Donor with Dengue Peptides**

Peptides	Amino acid sequences	Stimulation index‡ Peptide concentrations 1	0.1	0.01
			μg/ml	
D4.NS3.464-472	NPAQEDDQY	0.7	1.0	ND
D4.NS3.477-485	DPLKNDEDH	0.8	1.1	ND
D4.NS3.500-508	TPEGII PTL	2.6	2.7	1.8
D4.NS3.505-513	IPTLFGPER	0.9	0.8	ND
D4.NS3.510-518	GPEREKTQA	1.0	1.1	1.0
D4.NS3.542-550	LPVWLSYKV	1.2	1.1	ND
D4.NS3.592-600	RPRWLDARV	1.7	1.1	ND
D4.NS3.603-611	DPMALKDFK	1.3	0.8	ND
D4 Ag§				10.7
Control Ag				1.1

* PBMC (2×10^5 cells) were incubated for 7 d in the presence of peptides, dengue virus, or control antigen. 10% TCGF was added on day 3. Cells were pulsed with 1.5 μCi of [^{3}H]thymidine for 10 h, and [^{3}H]thymidine incorporation was measured. Stimulation indices of ≥2.0 are underlined.
‡ Stimulation index is the ratio of [^{3}H]thymidine incorporation (cpm) in the presence of peptides or antigens to [^{3}H]thymidine incorporation (cpm) in the absence of antigens. [^{3}H]thymidine incorporation without antigen was 14,970 cpm.
§ D4V antigen was prepared as described in Materials and Methods and used at a 1:120 dilution.

Table 2. *Recognition of D4.NS3.500-508 by CTLs Generated in Bulk Culture with D4V**

Autologous LCL infected or pulsed with	Percent of specific ^{51}Cr release‡ Experiment 1	Experiment 2
VV [D4:NS3]§	56	47
VV [control]	4	3
D4.NS3.464-472	0	0
D4.NS3.477-485	ND	0
D4.NS3.500-508	80	86
D4.NS3.505-513	ND	0
D4.NS3.510-518	ND	0
D4.NS3.542-550	ND	0
D4.NS3.592-600	ND	0
D4.NS3.603-611	0	0
None	0	ND

* 10^3 autologous LCL target cells were incubated with effector cells in the presence of peptides at 0.025 μg/ml for 5 h.
‡ Effectors were generated in 7-d bulk culture (Experiment 1) and 14-d bulk culture (Experiment 2). E/T cell ratios were 100:1 and 50:1 in Experiments 1 and 2, respectively.
§ VV [D4:NS3] indicates recombinant vaccinia virus that contains the gene coding for the NS3 protein of D4V.

and Methods (Table 3). Six of the eight peptides, including peptide D4.NS3.500-508, bound to HLA-B*3501, while two peptides did not bind. The results shown in Tables 1–3 suggest that the stimulation of the PBMC by D4.NS3.500-508 is caused, not only by the peptide's ability to bind to HLA-B35, but also by specific recognition of this peptide by CTLs.

Recognition of Dengue Virus NS3 Protein and the Peptide D4.NS3.500-508 by Dengue Virus–specific CD8⁺ CTL Clones. The PBMC from this D4V-immune donor were cultured with peptide D4.NS3.500-508 for 6 wk and was then subjected to limiting dilution to isolate CTL clones as described in Materials and Methods. Those CTL clones, which demonstrated D4V NS3–specific cytotoxicity in the screening CTL assays, were expanded and nine clones were selected. These nine CTL clones had a CD3⁺ CD4⁻ CD8⁺ phenotype, as determined by immunofluorescence staining.

After establishment, each of the nine CD8⁺ CTL clones was again examined for cytotoxicity against VV[D4:NS3]-infected and peptide D4.NS3.500-508–pulsed autologous LCL. All the tested CTL clones lysed VV[D4:NS3]-infected and peptide D4.NS3.500-508–pulsed target cells (Table 4). Recognition of two eight–amino acid peptides, D4.NS3.501-508 and D4.NS3.500-507, which are NH_2-terminal and COOH-terminal truncations of D4.NS3.500-508, respectively, was then examined. These two peptides were not recognized or were recognized to much lower levels compared to D4.NS3.500-508 by peptide-induced, dengue virus–specific CD8⁺ CTL clones (data not presented). This result indicates that the nine–amino acid peptide D4.NS3.500-508 represents the smallest recognition unit.

Lysis of Dengue Virus–infected Autologous LCL by CD8⁺ CTL Clones. To confirm that the CD8⁺ CTL clones established by stimulation with the peptide D4.NS3.500-508 lyse dengue virus–infected target cells, we examined the ability of the CTL clones 12 and 20–22 to lyse autologous LCL infected with D2V. These CTL clones lysed D2V–infected autologous LCL, but did not lyse uninfected LCL (Table 5).

Dengue Virus Serotype Specificities of CD8⁺ CD4⁻ CTL Clones. To determine dengue serotype specificities of the CD8⁺ CTL clones, we examined cytotoxicity against autologous LCL infected with VV [D2:NS3], VV [D3:NS3], or VV [D4:NS3] (Table 6). CTL clones 9 and 14 lysed target cells infected with VV [D4:NS3], but did not lyse target cells infected with other recombinant VV. Clone 12 lysed target cells infected with VV [D2:NS3] and VV [D4:NS3] and clones 2, 17, and 26 lysed target cells infected with VV [D3:NS3] and VV [D4:NS3]. Clones 20–22 lysed target cells infected with any of the three VV constructs. These results indicate that CD8⁺ T cell clones with four distinct patterns of dengue serotype specificities were established by stimulation

Table 3. *Quantitation of the Binding of Eight Peptides Synthesized from the Amino Acid Sequence of D4V NS3 to HLA-B*3501 Molecules**

Experiment	Peptide	Mean fluorescence intensity								
		Concentration of peptides (μM)								
		1,000	800	400	200	100	10	1	0.1	0.01
1	D4.NS3.477-485	-	143	84	63	48	40	40	37	36
	D4.NS3.500-508	-	219	197	194	180	82	46	40	37
	D4.NS3-505-513	-	140	97	55	36	38	42	41	38
	D4.NS3.542-550	-	156	144	97	53	41	40	40	38
	D4.NS3.592-600	-	24	36	41	45	45	47	41	41
	D4.NS3.603-611	-	208	191	142	77	45	41	38	37
2	D4.NS3.464-472	-	-	-	-	118	51	45	41	41
	D4.NS3.500-508	-	-	-	-	193	93	57	48	48
3	D4.NS3.510-518	47	-	-	-	46	45	52	47	48
	DR.NS3.500-508	327	-	-	-	210	113	60	49	49

* Peptide binding assays were performed using RMA-S-B*3501 cells as described in Materials and Methods. Mean fluorescence intensities without peptide were 37 in Experiment 1, 44 in Experiment 2, and 48 in Experiment 3.
The - symbol indicates concentrations not tested.

with a single viral peptide. The bulk-cultured T cell line that had been stimulated with D4.NS3.500-508 for 6 wk lysed target cells infected with VV[D2:NS3], VV[D3:NS3], or VV[D4:NS3].

We then synthesized peptides D2.NS3.500-508 (TPEGIIPSM) and D3.NS3.500-508 (TPEGIIPAL), which correspond to amino acids 500–508 of dengue 2 NS3 and dengue 3 NS3 respectively, to examine serotype specificities of the T cell clones at the peptide levels. The patterns of dengue virus serotype specificities determined using VV were con-

Table 4. *Recognition of Peptide D4.NS3.500-508–pulsed and VV[D4:NS3]-infected Autologous Target Cells by Dengue Virus–specific CD8+ T Cell Clones**

Dengue-specific clones	Percent of Specific ^{51}Cr release							
	Peptide D4.NS3.500-508 (μg/ml)				Control peptide (μg/ml)‡		No peptide	VV [D4:NS3]§
	2.5	2.5 × 10^{-2}	2.5 × 10^{-4}	2.5 × 10^{-6}	2.5	2.5 × 10^{-2}		
2	85	87	55	0	0	0	0	68
9	56	49	12	0	0	0	0	35
12	37	40	2	0	0	0	0	33
14	79	71	44	0	0	0	0	66
17	70	63	57	0	1	0	0	31
20	90	89	72	0	0	0	1	87
21	79	71	38	0	0	0	0	50
22	26	36	11	0	0	0	1	18
26	60	62	37	0	1	0	1	30

* A total of 103 autologous target cells were incubated with effector cells in the presence or absence of peptides for 5 h. E/T cell ratio was 10:1.
‡ D4.NS3.464-472 was used as a control peptide.
§ VV [D4:NS3] depicts rVV that contains genes coding for NS3 protein of D4V.

Table 5. *Lysis of Autologous LCL Infected with D2Vs by CD8+ CTL Clones**

CD8+ CTL clones	Percent of specific ^{51}Cr release	
	D2V-infected	Uninfected
12	30	0
20	59	6
21	67	0
22	34	0

* 10^3 target cells were incubated with effector cells for 4 h. D2V-infected and uninfected autologous LCL were used as target cells. E/T ratio was 10:1.

sistent with the serotype specificities determined at the peptide level (Table 7). The published sequence of amino acids 500–508 on NS3 is completely conserved between D3V and D1V viruses (29, 30). Therefore, we assume that the CTL clones that recognized D3V NS3 would also recognize D1V NS3.

HLA-B35 Restriction of Dengue Virus-specific CD8+ CTL Clones. HLA restriction of the lysis of target cells by the CD8+ T cell clones was examined using a panel of HLA-typed allogeneic LCL (Table 8). Seven CTL clones were tested, and they all lysed peptide D4.NS3.500-508–pulsed allogeneic LCL that shared HLA-B35, but they did not lyse D4.NS3.500-508–pulsed LCL that did not share HLA-B35. To confirm that our clones are HLA-B35 restricted, we also used a murine P815 cell line transfected with the HLA-B*3501 gene. Three clones, 14, 20, and 21, were tested and they lysed P815-B35 cells pulsed with peptide D4.NS3.500-508, but did not lyse P815-B35 cells pulsed with peptide D4.NS3.464-472 or cells not pulsed with any peptides (data not presented). These results indicate that the CD8+ T cell clones are HLA-B35 restricted.

Recognition of the Peptides with Amino Acid Substitution by Dengue Virus-specific CD8+ CTL Clones. To define the differences in the recognition of the peptide D4.NS3.500-508 by CD8+ CTL clones of different serotype specificities, we examined the effect of amino acid substitutions at single positions on recognition. Nine peptides that have aspartic acid (D) at one of the nine amino acid positions of D4.NS3.500-508 were synthesized. Three of the nine peptides, those that had D at the 2nd, 6th, or 7th position, did not bind to HLA-B*3501, but the other six substituted peptides bound (Table 9).

We examined these six substituted peptides (D4.NS3.500-500D, 502D, 503D, 504D, 507D, and 508D) that showed significant binding to HLA-B*3501 for recognition by CD8+ CTL clones in cytotoxicity assays (Table 10). The D4V specific

Table 6. *Dengue Serotype Specificities of Dengue Virus-specific CD8+ CTL Clones**

Dengue-specific clones	Percent of specific ^{51}Cr release			
	VV [D2:NS3]	VV [D3:NS3]	VV [D4:NS3]‡	VV [control]
Bulk culture§	44	81	69	5
Serotype-specific				
9	2	0	60	3
14	0	1	61	0
Subcomplex specific				
12	62	1	38	1
2	18	90	65	8
17	3	66	52	1
26	7	67	54	0
Serotype cross-reactive				
20	60	79	35	0
21	71	81	39	0
22	48	65	30	0

* 10^3 target cells were incubated with effector cells for 5 h. The E/T ratios were 8:1 for clone 20, and 10:1 for clones 2, 9, 12, 14, 17, 21, 22, and 26. The E/T ratio was 50:1 for bulk culture.
‡ VV [D2:NS3], VV [D3:NS3], and VV [D4:NS3] depict recombinant vaccinia viruses that contain genes coding for NS3 protein of D2V, D3V, and D4V, respectively.
§ PBMC were stimulated with the peptide D4.NS3.500-508 in bulk culture for 6 wk.

Table 7. *Serotype Specificities of Dengue Virus-specific CD8+ CD4- CTL Clones at the Peptide Levels**

	Percent of specific ^{51}Cr release of autologous targets with peptide‡					
	D2.NS3.500-508 (μg/ml)		D3.NS3.500-508 (μg/ml)		D4.NS3.500-508 (μg/ml)	
Dengue-specific clones	2.5	2.5×10^{-2}	2.5	2.5×10^{-2}	2.5	2.5×10^{-2}
Serotype specific						
9	0	0	2	0	61	35
14	0	0	0	0	76	60
Subcomplex specific						
12	23	0	1	0	52	69
2	20	1	57	11	90	85
17	0	0	23	6	69	73
Serotype cross-reactive						
20	68	66	78	64	84	88
21	71	69	59	46	66	63

* 10^3 autologous LCL target cells were incubated with effector cells in the presence of peptides at different concentrations (2.5 μg/ml, 2.5×10^{-2} μg/ml) for 5 h. E/T cell ratios were 10:1 percent specific ^{51}Cr release without peptide was 0% for clone 9, 0% for clone 14, 1% for clone 12, 0% of clone 2, 0% for clone 17, 0% for clone 20, and 0% for clone 21.
‡ Sequences of NS3:500-508 are TPEGIIPSL for D2V, TPEGIIPAL for D3V, and TPEGIIPTL for D4V.

clones 9 and 14 did not recognize peptides 503D, 504D, 507D, or 508D. The subcomplex-specific clones 12, 2, and 17 did not recognize 500D, 503D, or 507D, while the serotype–cross-reactive clones 20 and 21 did not recognize 503D. These results suggest that all of the CD8+ CTL clones of different serotype specificities recognize the original peptide D4.NS3.500-508, but the manner of recognition by these CTL clones is different, depending on serotype specificities of the clones.

Discussion

In this paper we characterized dengue virus–specific CD8+ CTL clones established by stimulation with the peptide D4.NS3.500-508. The peptide-stimulated, dengue virus–specific human CD8+ CD4- T cell clones were heterogeneous with a minimum of four patterns of serotype specificities: (*a*) D4V-specific; (*b*) cross-reactive for D1V, D3V, and D4V; (*c*) cross-reactive for D2V and D4V; and (*d*) cross-reactive for all four dengue virus serotypes. The serotype–cross-reactive clones 20, 21, and 22 were established from the wells that contained three cells per well; however, only 4% of the wells that originally contained three cells per well had the growing cells with specific cytotoxic activity. Analysis of TCR V gene usage by the previously reported PCR method (31) revealed that clones 20, 21, and 22 used a single TCR V gene, Vα8 and Vβ5.2. These results strongly suggest that these

Table 8. *Determination of HLA Class I Restriction of CD8+ CTL Clones Using Peptide-pulsed Allogeneic Target Cells**

	HLA class I type‡			Percent of specific ^{51}Cr release						
Target	A	B	C	2	9	12	14	17	20	21
Autologous	2/23	35/44	w4	88	84	39	84	65	85	58
JC	3/24	35	w4	80	87	35	84	86	84	55
TG	23/29	7/44	w4	6	7	0	3	4	16	6
M	24/30	13/55	w4/w6	6	11	0	3	1	24	5
VA 12	1/24	35	w4	85	37	19	20	28	68	26
VA 03	2/24	7/35	w3/w7	73	50	17	38	34	55	29

* A total of 10^3 targets were incubated with effector cells in the presence of peptide D4.NS3.500-508 (TPEGIIPTL) at 2.5×10^{-2} μg/ml for 5 h. The E/T cell ratio was 10:1 for clones 2, 9, 14, 20, 21, and 22, and 8:1 for clones 12 and 17.
‡ HLA loci that match donor HLA are underlined.

Table 9. *Quantitation of the Binding of Amino Acid Substituted D4.NS3.500-508 Peptides to HLA-B*3501 Molecules**

Peptides[b]	Amino acid sequences	Mean fluorescence intensity					
		Concentration of peptides (μM)					
		1,000	100	10	1	0.1	0.01
D4.NS3.500-508	TPEGIIPTL	316	253	125	74	76	64
D4.NS3.500.500 D	DPEGIIPTL	311	190	88	70	65	67
D4.NS3.500.501 D	TDEGIIPTL	74	72	74	71	66	71
D4.NS3.500.502 D	TPDGIIPTL	266	125	74	69	71	69
D4.NS3.500.503 D	TPEDIIPTL	373	301	177	100	73	71
D4.NS3.500.504 D	TPEGDIPTL	273	147	78	70	67	63
D4.NS3.500.505 D	TPEGIDPTL	70	62	69	64	71	71
D4.NS3.500.506 D	TPEGIIDTL	83	73	65	67	70	71
D4.NS3.500.507 D	TPEGIIPDL	328	202	71	69	72	62
D4.NS3.500.508 D	TPEGIIPTD	134	66	64	66	65	69

* Peptide binding assays were performed using RMA-S-B*3501 cells as described in Materials and Methods. The mean fluorescence intensity in the absence of peptide was 69. Peptides tested included a series of peptides with aspartic acid substitutions indicated by the underlined D in the sequence.

clones represent individual clones with broad reactivity rather than mixture of the clones. Furthermore, there were clones 2 and 10 that were established from the wells that originally contained one cell per well, and that had the same serotype cross-reactivity as clones 20–22. Unfortunately, clones 2 and 10 did not grow enough to be used throughout the entire experiments (data not presented).

Serotype specificities determined using a rVV were consistent with those determined using synthetic peptides. We used dengue-vaccinia recombinant viruses that contained genes for the NS3 of D2V, D3V, or D4V, and the peptides that corresponded to amino acids 500–508 on NS3 proteins of D2V, D3V, and D4V. Peptides D2.NS3.500-508 and D3.NS3.500-508 have some amino acid substitutions as compared to the D4V peptide. Peptide D2.NS3.500-508 has two amino acid substitutions; S for T at amino acid 507 and M for L at amino acid 508 (32, 33). D1V and D3V have the same amino acids sequences at amino acids 500–508, and have one amino acid substitution as compared to D4V; A for T at amino acid 507 (29, 30, 32). CTLs cross-reactive for D4V and D2V recognized peptides D2.NS3.500-508, and those cross-reactive for D4V and D3V recognized D3.NS3.500-508.

The serotype specificities of $CD8^+$ CTL clones are determined by the interaction of the TCR with peptides that are bound to HLA. The amino acids at the 2nd and 9th positions of the peptide are reported to be residues that are important for binding to HLA-B35 (24). Amino acids 500–506 are completely conserved among D1V–D4V. Thus, if we hypothesize that TCR of CTL clones of heterogeneous serotype specificities recognize the amino acids at the same position of the peptide as critical residues, amino acid 507 may be critical for recognition by these CTL clones. The results suggest that the TCRs of dengue 4–specific and subcomplex-specific CTL clones may be very specific and distinguish the difference of amino acids between D4V and other serotypes of dengue viruses, whereas the TCRs of CTL clones 20–22 do not discriminate this difference. Alternatively, TCRs of serotype–cross-reactive CTL clones 20–22 may recognize between amino acids 500 and 506 which are completely conserved among the four dengue serotypes, as the critical residues.

The other possibility is that the critical residues for TCR binding are the same for all of the CTL clones and are conserved among four serotypes of dengue viruses, but are exposed differently on HLA-B35 (34). For example, the binding

Table 10. *Recognition of Amino Acid Substituted D4.NS3.500-508 Peptides by Dengue Virus-specific CD8+ CTL Clones**

Dengue-specific clones	Percent relative lysis compared to D4.NS3.500-508					
	500‡	502D	503D	504D	507D	508D
Serotype specific						
9	67	70	9	9	2	36
14	87	87	4	17	0	20
Subcomplex specific						
12	45	121	12	97	0	112
2	23	107	20	91	0	70
17	8	72	25	64	1	83
Serotype cross-reactive						
20	23	94	9	76	47	94
21	63	75	13	46	100	90

* Percent of relative lysis compared to D4.NS3.500-508 was calculated by the formula: 100 × (percent of specific ^{51}Cr release with or substituted peptide at 25 μg/ml)/(percent of specific ^{51}Cr release with D4.NS3.500-508 at 25 μg/ml). Percent of specific ^{51}Cr release with D4.NS3.500-508 was: 74% (clone 2), 69% (clone 9), 33% (clone 12), 77% (clone 14), 36% (clone 17), 87% (clone 20), and 24% (clone 21) for 500D, 502D, 503D and 504D; and 91% (clone 2), 76% (clone 9), 76% (clone 12), 60% (clone 14), 88% (clone 17), 80% (clone 20), and 78% (clone 21) for 507D and 508D.
‡ The amino acid sequences of the substituted peptides were 500D, DPEGIIPTL; 502D, TPDGIIPTL; 503D, TPEDIIPTL; 504D, TPEGDIPTL; 507D, TPEGIIPDL; 508D, TPEGIIPTD.

of D3.NS3.500-508 to HLA-B35 may be different from that of D4.NS3.500-508 because of the substitution of the amino acid at the 8th position. It is possible that serotype–cross-reactive CTL clones recognize the critical residues on both of the peptides bound to HLA-B35, while D4V-specific CTL clones can recognize the critical residues on D4.NS3.500-508, but cannot recognize the same critical residues on D3.NS3.500-508 bound to HLA-B35.

To further understand the interaction of TCRs with critical amino acid residues of the peptide, we prepared nine peptides by substituting each of the nine amino acids of the D4.NS3.500-508 with aspartic acid. The recognition of these substitutions by the $CD8^+$ CTL clones varied depending on the serotype specificities of the clones. The peptide D4.NS3.500.503D, which has an aspartic acid substitution at the 4th position, was not recognized by any of the clones, although this peptide bound to HLA-B35, as well as the original peptide D4.NS3.500-508. The peptide D4.NS3.500.504D was not recognized by the D4V-specific CTL clones, but was recognized by the subcomplex-specific and serotype–cross-reactive CTL clones. The peptide D4.NS3.500.507D was not recognized by the D4V-specific or subcomplex-specific CTL clones, but was recognized by the serotype–cross-reactive clones. These results are consistent with the reports that the TCR has contact with more than one amino acid residue of an antigenic peptide bound to the HLA (35, 36), and that glutamic acid at the 4th position, which is conserved among the four serotypes of dengue viruses, may be the most critical residue for recognition. These results also suggest that the differences in the recognition of the D2V, D3V, and D4V peptides may be caused by the differences in the configuration of these peptides when they are bound to HLA. On the other hand, recognition of D4.NS3.500.507D by serotype–cross-reactive clones, but not by D4V-s or subcomplex-specific clones, suggest that the critical residue for recognition may be different among T cells of different serotype specificities. Further experiments are necessary to delineate the interactions of the TCR of these CTL clones and dengue peptides.

Dengue virus infections are a serious cause of human morbidity and mortality in tropical and subtropical areas of the world (37, 38). The pathological mechanisms, which result in a severe complication of some dengue virus infections, termed dengue hemorrhagic fever (DHF), are not understood. Patients with DHF are usually undergoing the secondary infection with a different serotype of dengue virus than that which caused the primary infection (39). Patients with DHF have highly activated $CD8^+$ and $CD4^+$ T cells (40). The presence of activated $CD8^+$ CTL during a secondary dengue virus infection raises the possibility that these CTL may contribute to the pathogenesis of DHF through lymphokine production and lysis of dengue virus–infected monocytes. On the other hand, a strong dengue virus–specific CTL response is probably necessary for recovery from dengue virus infection. Currently, there is no effective vaccine against dengue virus infections, although live attenuated experimental vaccines that induce immune responses in humans have been de-

veloped (41, 42). Putative vaccine(s) should induce strong protective immune responses, but should not lead to the pathogenesis of DHF. Definition of the epitopes recognized by dengue virus-specific CD8+ CTL provides information that is useful for the development of safe and effective vaccines.

We have shown that a single nine-amino acid peptide induced virus-specific CTLs with heterogeneous serotype specificities from PBMC of a dengue virus-immune donor. The priming with live attenauted virus in vivo, however, may have had a profound effect on the precursor pool of CTL, and primary vaccination with the nine-amino acid peptide may give significantly different results. Synthetic peptides have not yet been successfully developed as human vaccines. The strategy presented in this paper addresses virus heterogeneity of CTLs induced by a single peptide, but is still subject to the constraints of HLA heterogeneity. Therefore, immunization with a subunit vaccine containing a single T cell epitope is not a realistic approach to inducing broad immunity in an outbred population.

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