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Identification of Amino Acids Involved in Recognition by Dengue Virus NS3-Specific, HLA-DR15-Restricted Cytotoxic CD4$^+$ T-Cell Clones

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The majority of T-cell clones derived from a donor who experienced dengue illness following receipt of a live experimental dengue virus type 3 (DEN3) vaccine cross-reacted with all four serotypes of dengue virus, but some were serotype specific or only partially cross-reactive. The nonstructural protein, NS3, was immunodominant in the CD4$^+$ T-cell response of this donor. The epitopes of four NS3-specific T-cell clones were analyzed. JK15 and JK13 recognized only DEN3 NS3, while JK44 recognized DEN1, DEN2, and DEN3 NS3 and JK5 recognized DEN1, DEN3, and West Nile virus NS3. The epitopes recognized by these clones on the DEN3 NS3 protein were localized with recombinant vaccinia viruses expressing truncated regions of the NS3 gene, and then the minimal recognition sequence was mapped with synthetic peptides. Amino acids critical for T-cell recognition were assessed by using peptides with amino acid substitutions. One of the serotype-specific clones (JK13) and the subcomplex- and flavivirus-cross-reactive clone (JK5) recognized the same core epitope, WITDFVGKTVW. The amino acid at the sixth position of this epitope is critical for recognition by both clones. Sequence analysis of the T-cell receptors of these two clones showed that they utilize different Vβ chains. The core epitopes for the four HLA-DR15-restricted CD4$^+$ CTL clones studied do not contain motifs similar to those proposed by previous studies on endogenous peptides eluted from HLA-DR15 molecules. However, the majority of these dengue virus NS3 core epitopes have a positive amino acid (K or R) at position 8 or 9. Our results indicate that a single epitope can induce T cells with different virus specificities despite the restriction of these T cells by the same HLA-DR15 allele. This finding suggests a previously unappreciated level of complexity for interactions between human T-cell receptors and viral epitopes with very similar sequences on infected cells.

Dengue viruses are members of the family *Flaviviridae* and are transmitted to humans predominantly by *Aedes aegypti* and *Aedes albopictus* mosquitoes (30). Molecular epidemiologic studies have shown that the dengue viruses in forest cycles are distinct from the viruses circulating between humans and mosquitoes in urban transmission cycles (32). Although sporadic pandemics of dengue fever are known to have occurred at intervals of up to 30 years over the past 200 years, the more severe form of disease, dengue hemorrhagic fever (DHF), was recognized only after multiple dengue virus serotypes had established overlapping hyperendemic transmission patterns in urban areas around the Pacific following World War II (30). Since then, endemic transmission cycles with multiple serotypes of dengue virus have been established in regions of Asia, Africa, and the Americas, and dengue virus has now become the leading cause of arthropod-borne virus disease in humans (11, 30). The number of annual dengue fever cases worldwide is estimated to be more than 100 million, with 250,000 reported cases of DHF. The true incidence of both diseases is estimated to be severalfold higher (30). Patients with DHF first show symptoms typical of dengue fever but then rapidly develop thrombocytopenia, diffuse capillary leakage, hemoconcentration, and hypotension. In the most severe form of the disease, dengue shock syndrome, the pulse pressure narrows and circulatory failure can occur. Supportive treatment has reduced case fatality rates from 20% to 1% (30).

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Dengue viruses contain an 11-kb single-stranded RNA genome of positive polarity and are divided into four antigenically distinct serotypes, designated types 1, 2, 3, and 4 (7). Comparison of the genomic sequences of different dengue virus serotypes revealed a 65 to 75% nucleotide and a 67 to 75% amino acid homology (10). Dengue viruses within a serotype vary from each other by about 10% at the nucleotide level and by about 4% at the amino acid level (32, 36). Dengue viruses predominantly infect mononuclear phagocytes (13).

Children are the most susceptible hosts for DHF. In some areas of the world, DHF is now the third or fourth most frequent cause of hospitalization of children (14). DHF is most often associated with a secondary dengue infection caused by a dengue virus serotype different from that causing the primary infection. Serotype-cross-reactive antibodies induced during primary dengue virus infection are rapidly produced during secondary infection and form infectious immune complexes with virus. These virus-antibody complexes can efficiently infect monocytes via the Fc receptor, a phenomenon termed immune enhancement, which has been hypothesized to cause an increase in the number of infected cells and the yield of virus (12, 13). Marked activation of both CD4$^+$ and CD8$^+$ dengue virus serotype-cross-reactive cytotoxic T cells occurs in humans infected with dengue virus (19, 22–25). Elevated levels of soluble CD4$^+$ were found in children with dengue infection and were highest in children with DHF (23). The levels of soluble CD4$^+$ are indicative of the level of CD4$^+$ T-cell activation.

The host- and virus-specific factors that determine why a

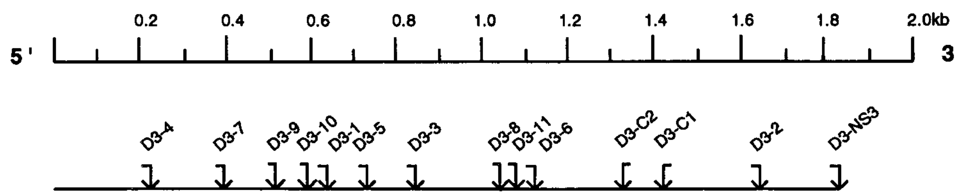

FIG. 1. Schematic representation of the truncated NS3 proteins expressed by recombinant VV. Bent arrows on the NS3 gene represent the truncation sites. D3-C1 and D3-C2 are C-terminal fragments; all others are N-terminal fragments.

secondary dengue virus infection in one individual causes no symptoms or dengue fever but DHF develops in another individual are currently not well understood. Also, to date, there is no animal model for DHF. Both serotype-specific and serotype-cross-reactive $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ dengue virus-specific memory T cells have been obtained from human donors immunized with a single-serotype, live, attenuated, experimental dengue virus vaccine (4, 19). In bulk culture, these T cells respond to stimulation by each of the four dengue virus serotypes but always show the highest response to the homologous serotype. Similar results were also obtained with murine memory T cells derived from animals immunized with a single serotype of dengue virus (33). For about 50% of the human donors tested thus far, the nonstructural protein NS3 is the immunodominant dengue virus protein, while the viral envelope protein is immunodominant for the others (21). The NS3 protein contains an N-terminal protease domain and a C-terminal helicase domain (5, 7).

The T-cell clones used in this study were isolated from a donor who had been immunized with yellow fever virus (YFV) vaccine and then with an experimental dengue virus type 3 (DEN3) vaccine. Most of the T-cell clones isolated from this donor were dengue virus serotype cross-reactive (19). To identify the amino acids involved in restricted recognition by serotype-specific and subcomplex-cross-reactive T-cell clones, two DEN3-specific, one dengue virus subcomplex-cross-reactive, and one dengue virus subcomplex- and flavivirus-cross-reactive clone were selected for epitope mapping. Amino acid sequences involved in recognition by these four T-cell clones were determined by using vaccinia virus (VV) recombinants and overlapping synthetic peptides.

MATERIALS AND METHODS

Cells. Vero cells were obtained from the American Type Culture Collection and used to grow the four serotypes of dengue virus. CV-1 and 143 B cells were provided by Paul Rota, Centers for Disease Control and Prevention, and were used for recombinant VV production. These three cell lines were cultured in minimal essential medium containing 5 or 10% fetal calf serum. A lymphoblastoid cell line (B cell) was established by Epstein-Barr virus transformation of homologous peripheral blood mononuclear cells obtained from the donor and cultured in RPMI containing 10% fetal calf serum as described previously (19).

Establishment of dengue virus-specific human T-cell clones. The details of the procedures used to establish the four T-cell clones described in this report were previously described in detail (19, 24). Peripheral blood mononuclear cells (4×10^5) were cultured for 7 days with dengue virus antigen at a final dilution of 1:30 in 0.2 ml of RPMI containing 10% human AB serum in 96-well round-bottom plates. On day 7, blast cells were enriched by Ficoll-Hypaque density gradient centrifugation. The JK5, JK13, and JK15 clones were established by culturing blast cells at a concentration of three cells per well in the presence of γ-irradiated autologous peripheral blood mononuclear cells (10^5) in 0.2 ml of RPMI containing 10% human AB serum, 10% interleukin-2, and dengue virus antigen at a final concentration of 1:30. The JK44 clone was established by using 10 cells per well. The JK5, JK13, and JK15 clones were established in the presence of DEN3 antigen, while the JK44 clone was established with DEN2 antigen. PCR analysis of the Vβ chains of the T-cell receptor (TCR) detected only a single Vβ band for each of these four clones (data not shown). In addition, a single Vα band was detected for two of the clones, JK5 and JK15. These data indicate that each of these four T-cell lines is a clone.

Viruses. DEN1 (Hawaii strain) and DEN2 (New Guinea C strain) were obtained from Walter E. Brandt, Walter Reed Army Institute of Research, Washington, D.C. DEN3 (CH 53489 strain) was provided by Bruce L. Innis, Armed Forces Research Institute of Medical Science, Bangkok, Thailand, and DEN4 (814669 strain) was provided by Jack McCown, Walter Reed Army Institute of Research. YFV (17D strain) was supplied by Jacob J. Schlesinger, University of Rochester School of Medicine and Dentistry. West Nile virus (WNV) was obtained from J. S. Porterfield. VV (Wyeth strain) was provided by Paul Rota. Stocks of dengue virus and YFV were propagated in C6/36 cells. Stocks of WNV and VV were propagated in BHK and CV-1 cells, respectively.

Preparation of dengue virus antigens. Dengue virus antigens, YFV antigen, and WNV antigen were prepared from infected Vero cells as described previously (19), using virus-infected cytoplasmic supernatant obtained from the cell extract by centrifugation at $2,000 \times g$ for 10 min. The control antigen was prepared in the same manner, using uninfected Vero cells. All antigen preparations were in a volume of 15 ml and were from 15 T-75 flasks (Corning) of confluent Vero cells.

Construction of VV recombinants. VV recombinants expressing DEN2 NS3 or DEN3 NS3 were generated as described previously (28). Briefly, DEN2 and DEN3 genomic RNAs were extracted from virions partially purified by sedimentation through a 5, 10, and 20% discontinuous glycerol gradient made with TNE buffer (50 mM Tris-HCl [pH 7.2], 100 mM NaCl, 1 mM EDTA). The NS3 genes of DEN2 and DEN3 were amplified from their respective genomic RNAs by reverse transcription-PCR, using forward and reverse 20-mer primers located at the 5′ and 3′ ends of the gene. The 5′ primer contained a *Bam*HI site and an in-frame AUG start codon immediately preceding the NS3 N-terminal coding sequence, while the 3′ primer contained a UAA stop codon followed by a *Hin*dIII site. Neither a *Bam*HI site nor a *Hin*dIII site is present in the NS3 gene. The PCR cycle used was 94°C for 1 min, 42°C for 20 s, and 72°C for 3 min for a total of 30 cycles. PCR products were cloned into the VV transfer plasmid pKG19 at the *Bam*HI and *Hin*dIII restriction sites. The junction sequences of the recombinant plasmids produced were confirmed by dideoxy sequencing (35). Recombinant plasmid pKG19-D3NS3 (5 μg) or pKG19-D2NS3 (5 μg) and wild-type VV DNA (1 μg) were transfected into CV-1 cells in the presence of Lipofectin (25 μg; Bethesda Research Laboratories). At the time of transfection, CV-1 cells were 75% confluent and had been infected with VV for 2 h at a multiplicity of infection of 0.01. VV recombinants were plaque purified on 143 B cells (thymidine kinase negative) in the presence of bromodeoxyuridine (0.3 mg/ml), amplified in CV-1 cells in 24-well plates, and resuspended in 300 μl of PBS-M buffer (phosphate-buffered saline supplemented with 0.1 M $MgSO_4$). The virus sample (10 μl) was diluted 10-fold with buffer containing 10 mM Tris-HCl (pH 8.5), 1% Nonidet P-40, and 2 mg of protease K per ml and incubated at 65°C for 2 h. After being heated at 100°C for 10 min to inactivate the protease, cell lysates (5 μl for each) were used as templates for PCR to confirm the presence of the insert in the recombinant. The forward and reverse primers were located at the 5′ and 3′ ends of the inserted gene. Recombinant viruses VVD2-NS3 and VVD3-NS3 were plaque purified two additional times in the presence of bromodeoxyuridine before further use.

VV recombinants expressing various N-terminally or C-terminally deleted versions of DEN3 NS3 were constructed by a similar procedure. Figure 1 shows the truncation sites. The various C-terminally deleted fragments of the DEN3 NS3 gene were produced by PCR amplification from pKG19-D3NS3 plasmid DNA, using the genomic sense DEN3 NS3 gene 5′ primer described above for amplifying the entire DEN3 NS3 gene and different antisense 3′ primers. The 3′ primers contained a stop codon and a *Hin*dIII site. Two N-terminally deleted NS3 fragments were also generated by PCR, using the genomic antisense DEN3 NS3 gene 3′ primer described above for amplifying the entire DEN3 NS3 gene and different genomic sense primers containing a *Bam*HI restriction site and an AUG codon. All of the primers were 18 to 21 nucleotides in length. PCR amplification of the truncated NS3 fragments was performed under the conditions used for the complete NS3 gene except that the extension time was reduced by 1 or 2 min as the length of the template decreased.

Synthetic peptides and oligonucleotides. Peptides were synthesized with an automated model 431A peptide synthesizer (Applied Biosystems). The N-terminal 9-fluorenylmethoxycarbonyl group was removed from peptides on the machine. Peptides were deprotected and cleaved from the resin according to the procedure suggested by the manufacturer. Oligonucleotides were synthesized on a model 384 DNA synthesizer (Applied Biosystems).

Cytotoxic T-lymphocyte (CTL) assay. Target B-lymphoblastoid cells were infected with recombinant VV or pulsed with viral antigen as described previously (4, 19). A total of 10^6 target cells were infected with recombinant or wild-type VV at a multiplicity of infection of 10 to 20 or incubated with virus antigen diluted 1:80. After overnight incubation, target cells were labeled with 500 μCi of $Na_2{}^{51}CrO_4$ (Dupont) at 37°C for 45 min and washed three times with RPMI–10% fetal calf serum.

The procedures used for peptide pulsation were similar to those used for viral antigen described above (20). Cells were labeled with ^{51}Cr, and then 10^3 cells in 0.1 ml were pulsed with 50 μl of peptide at a concentration of 25, 2.5, 0.25, 0.025, or 0.0025 μg/ml for 30 min at 37°C.

Labeled target cells (0.1 ml) were added to each well of a round-bottom microtiter plate (Falcon Plastic) at a concentration of 10^4 cells per ml. CTLs were then added to each well in various concentrations to give different effector/target ratios. CTLs were added in a volume of 0.1 ml for viral antigen and VV experiments and in a volume of 50 μl for peptide experiments. The CTL assay was carried out for 4 h at 37°C, and the supernatant fluids were collected from each well with a Titertek harvester (Skatron Instruments, Lier, Norway). Released ^{51}Cr was measured with a gamma counter, and the percentage of specific ^{51}Cr release was calculated with the following formula: 100 × (cpm experimental release − cpm spontaneous release)/[cpm maximal release (detergent lysis) − cpm spontaneous release]. Background levels of spontaneous ^{51}Cr release from labeled target cells were usually less than 25%.

Determination of the HLA restriction patterns of the T-cell clones. Monoclonal antibody (MAb) B7/21.7, which detects HLA-DP, and MAb S3/4, which detects HLA-DQ, were kindly provided by Nancy Reinsmoen, University of Minnesota. MAb OKIa1 was purchased from Ortho Diagnostic Systems, Inc., Raritan, N.J. To analyze the HLA restriction pattern of JK44, 0.05 ml of a 1:20 dilution of MAb was added to ^{51}Cr-labeled target cells, and cells were pulsed with peptide 44b added to a final concentration of 2.5 μg/ml. These cells were then used in the CTL assay (20).

In the second method used for HLA typing, autologous target B-lymphoblastoid cells were replaced with 3107, 3161, 9038, MS, or VA-9 cells in the cytotoxicity assay. Autologous B-lymphoblastoid cells and the allogeneic target cells, MS and VA-9, had been previously HLA typed in the University of Massachusetts Medical Center Tissue Typing Laboratory. The other cell lines were purchased from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, N.J.). Peptide 13-5d (0.25 μg/ml) was used to coat target cells for HLA typing studies with both the JK13 and JK5 clones. Peptides 15a (0.25 μg/ml) and 44b (2.5 μg/ml) were used for studies of the JK15 and JK44 clones, respectively.

Sequencing of DEN1 (Hawaii strain) within the JK5 recognition region. Supernatant from DEN1 (Hawaii strain)-infected C6/36 cells was collected at 8 days after infection at a multiplicity of infection of 0.1. Virus was pelleted through a discontinuous glycerol gradient, and viral RNA was extracted as described above. Reverse transcription-PCR was performed with 18-mer primers to amplify the region located between nucleotides 936 and 1165 of the DEN1 NS3 gene (10). The PCR cycle was 94°C for 1 min, 42°C for 20 s, and 72°C for 2 min. A total of 30 cycles were run. The resulting PCR product was gel purified, cloned into the pCRII vector (Invitrogen, Inc.), and sequenced.

Determination of Vβ chain usage in the TCR. Total cytoplasmic RNA was extracted from 5×10^5 cells by the acid guanidinium-phenol-chloroform method (9). The RNA was treated with DNase I containing RNA Guard (Pharmacia Biotec, Inc.). First-strand cDNA was synthesized from this RNA by using random hexamers (Pharmacia Biotec) as primers and a Moloney murine leukemia virus reverse transcriptase (Gibco-BRL Life Technologies, Inc.). PCR was then performed with 1 of 26 5′ primers located in the variable region of the β chain and a 3′ primer located in the constant region of the β chain. The 3′ primer was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Promega Corp.) and purified on a Chroma Spin-10 column (Clontech Laboratories, Inc.). Labeled primer (10^5 cpm) was added to each PCR mixture. The thermal cycle was 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A total of 26 cycles were carried out. In the first cycle, a longer denaturation time (1.5 min) was used. PCR products were electrophoresed on a 5% polyacrylamide gel. Radioactivity in each PCR fragment was quantitated with a Betascope (Betagen).

Both strands of the PCR products were sequenced by using a double-stranded DNA cycle sequencing system (Gibco-BRL Life Technologies) and ^{32}P-labeled primers.

RESULTS

Virus specificities of the T-cell clones. $CD4^+$ $CD8^-$ T-cell clones were established from a donor previously immunized with YFV vaccine and then with a live, attenuated, experimental DEN3 vaccine (19, 25). To analyze the virus specificities of four of these clones, antigens from the four dengue virus serotypes, YFV, or WNV were incubated with autologous Epstein-Barr virus-transformed B-lymphoblastoid cells, and the T-cell clones were examined for the ability to lyse the antigen-pulsed target cells (Table 1). The JK13 and JK15 T-cell clones lysed target cells pulsed with DEN3 virus antigen but did not lyse target cells pulsed with DEN1, -2, or -4, YFV, or WNV antigen. Therefore, JK13 and JK15 are DEN3-specific clones.

TABLE 1. Recognition of virus antigens by four $CD4^+$ $CD8^-$ T-cell clones

Clone	% Specific ^{51}Cr release[a] for target cells with:							
	DEN1	DEN2	DEN3	DEN4	YFV	WNV	Control antigen	No antigen
JK15	0	0	<u>51</u>	0	0	0	0	0
JK13	1	1	<u>32</u>	0	1	1	2	2
JK5	<u>41</u>	1	<u>85</u>	2	0	<u>41</u>	1	2
JK44	<u>29</u>	<u>50</u>	<u>59</u>	1	0	0	4	0

[a] Vero cell lysates infected with one of the four serotypes of dengue virus, YFV, or WNV were used as virus antigens to coat target B cells for CTL assays. An uninfected Vero cell lysate was used as the control antigen. Values representing significant levels of lysis are underlined. The CTL assay time was 4 h, and the effector/target ratios were 20 for JK15, 11 for JK13, and 10 for JK5 and JK44.

JK5 lysed target cells pulsed with DEN1 or -3 or WNV antigen but did not lyse target cells pulsed with DEN2 or -4 or YFV antigen. Therefore, JK5 is dengue virus subcomplex and flavivirus subcomplex specific. This pattern of virus cross-reactivity had not been observed in previous studies of human dengue virus-specific T-cell clones (19). Repeated analysis of the virus specificity of the JK5 clone consistently confirmed the results in Table 1. The JK44 clone lysed target cells coated with DEN1, -2, or -3 antigen but did not lyse target cells coated with DEN4, YFV, or WNV antigen. This result is consistent with previous data (19) indicating that JK44 is dengue serotype subcomplex specific.

Recognition of VV-expressed dengue NS3 protein. As described previously (19), most of the analyzed dengue virus-cross-reactive JK T-cell clones, including JK44, recognize the dengue virus NS3 protein. T-cell clone reactivity was detected by using an available DEN4 NS3 protein expressed by VV or an NS3 protein isolated from DEN3-infected cells (19). To determine whether the new clones, JK5, JK13, and JK15, recognized the NS3 protein, we prepared two additional VV recombinants, VVD2-NS3 and VVD3-NS3, which expressed DEN2 NS3 and DEN3 NS3, respectively, and used these constructs to infect target cells for cytotoxicity assays. All four of the T-cell clones recognized NS3. Three of the clones, JK15, JK13, and JK5, recognized target cells infected with VVD3-NS3 but did not recognize target cells infected with VVD2-NS3 or VV. JK44 recognized target cells infected with either VVD2-NS3 or VVD3-NS3 (data not shown). None of the clones recognized target cells infected with nonrecombinant VV. The virus specificities detected with antigen expressed by VV were consistent with those observed with virus-infected cell extracts (Table 1).

Mapping the T-cell epitopes by using truncated DEN3 NS3 peptides expressed by VV recombinants. VV was used to express various terminally truncated fragments of NS3. The NS3 gene was truncated at six positions to produce five N-terminal and one C-terminal NS3 fragments. VV recombinants expressing each of these truncated fragments were generated and designated according to the order in which they were produced. The VV recombinants were used to infect target B cells, which in turn were used to examine the cytotoxic activity of each T-cell clone. We then made seven additional VV recombinants which expressed NS3 fragments that were truncated within the recognition regions for each clone identified with the first set of NS3 fragments (Fig. 1).

TABLE 2. Recognition of recombinant VV by CD4$^+$ CD8$^-$ T-cell clones

Clone	VV	aa[a]	% Specific ^{51}Cr release[b]				
			Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
JK15	VVD3-NS3	1–618	47	17	45	—	—
	VVD3-2	1–548	—	34	32	—	—
	VVD3-3	1–283	32	64	34	—	—
	VVD3-5	1–245	—	—	0	—	—
	VVD3-1	1–214	8	—	2	—	—
	VV		4	5	6	—	—
JK44	VVD3-NS3	1–618	—	—	—	87	—
	VVD3-2	1–548	70	78	—	—	—
	VVD3-3	1–283	67	55	—	—	—
	VVD3-5	1–245	54	58	—	—	—
	VVD3-1	1–214	—	66	77	86	59
	VVD3-10	1–205	—	—	—	—	4
	VVD3-9	1–176	—	—	—	1	3
	VVD3-7	1–133	—	—	1	—	—
	VVD3-4	1–83	—	2	0	—	—
	VVD3-C1	447–618	9	5	—	—	—
	VV		2	2	6	2	0
JK13	VVD3-NS3	1–618	29	64	—	—	—
	VVD3-2	1–548	27	56	—	—	—
	VVD3-6	1–374	—	78	25	49	—
	VVD3-11	1–354	—	—	—	16	—
	VVD3-8	1–340	—	—	5	21	—
	VVD3-3	1–283	3	—	—	—	—
	VVD3-5	1–245	—	—	0	—	—
	VVD3-1	1–214	8	—	—	19	—
	VVD3-C1	447–618	—	10	—	—	—
	VV		0	7	1	11	—
JK5	VVD3-NS3	1–618	67	65	—	—	—
	VVD3-2	1–548	33	41	—	—	—
	VVD3-6	1–374	—	49	56	36	—
	VVD3-11	1–354	—	—	—	6	—
	VVD3-8	1–340	—	—	9	—	—
	VVD3-3	1–283	0	5	—	—	—
	VVD3-5	1–245	—	—	7	—	—
	VVD3-C1	447–618	—	8	—	—	—
	VVD3-C2	412–618	—	4	—	—	—
	VV		6	8	5	1	—

[a] Amino acid (aa) regions on NS3 expressed by the various VV recombinants.
[b] Significant levels of lysis are underlined. Experiments not done are indicated by dashes. The assay time was 4 h for all experiments. Effector/target ratios were 18, 6, and 20 for experiments 1, 2, and 3 with JK15, respectively; 3, 20, 4, and 15 for experiments 1, 2, 3, and 4 with JK13, respectively; 5, 20, 12, and 7 for experiments 1, 2, 3, and 4 with JK5, respectively; and 13, 13, 20, 10, and 11 for experiments 1, 2, 3, 4, and 5 with JK44, respectively.

JK15 lysed target cells infected with the positive control VVD3-NS3, VVD3-2, or VVD3-3 but not with VVD3-1, VVD3-5, or the wild-type VV negative control (Table 2). HLA class II core epitope sequences usually contain 9 to 12 amino acids. If the D3-5 truncation was made within the core sequence, JK15 would not be able to recognize the truncated fragment. Therefore, the possible recognition region for JK15 was extended by 12 amino acids at the N-terminal end of the D3-5 truncation point. The JK15 recognition region in NS3 was thus localized between amino acids 234 and 283.

JK44 lysed target cells infected with VVD3-NS3, VVD3-1, VVD3-2, VVD3-3, or VVD3-5 but did not lyse target cells infected with VVD3-4, VVD3-7, VVD3-9, VVD3-10, or VVD3-C-1 (Table 2). Thus, the epitope recognized by JK44 was located between amino acids 194 and 214.

JK13 lysed target cells infected with VVD3-NS3, VVD3-2, or VVD3-6 but not with VVD3-1, VVD3-3, VVD3-5, VVD3-8, VVD3-11, VVD3-C1, or VV (Table 2). Similarly,

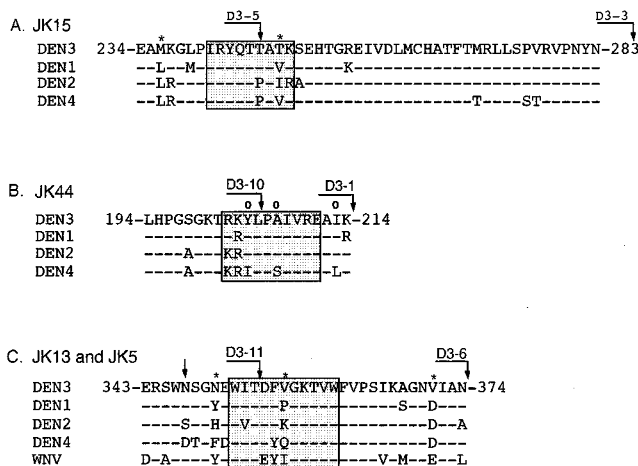

FIG. 2. Alignment of the amino acid sequences of the four dengue virus serotypes and WNV within the T-cell recognition regions mapped by truncated NS3 fragments expressed by recombinant VV. Sequences are from references 31 (for DEN3 [H87 strain]), 10 (for DEN1 [Singapore strain]), 18 (for DEN2 [NGC strain]), 29 (for DEN4 [814669 strain]), and 2 (for WNV). The location of the region on DEN3 NS3 is indicated by numbers at the N and C termini of each amino acid sequence. Identical amino acids are indicated by dashes. Asterisks represent DEN3 unique amino acids. The circles represent amino acids common in DEN1, DEN2, and DEN3. The arrowhead represents an amino acid common to DEN1, DEN3, and WNV. Arrows represent the truncation sites of the indicated NS3 fragments expressed by recombinant VV.

JK5 lysed target cells infected with VVD3-NS3, VVD3-2, or VVD3-6 but did not lyse target cells infected with VVD3-3, VVD3-5, VVD3-8, VVD3-11, VVD3-C1, or VVD3-C2 (Table 2). Both JK13 and JK5 recognized an epitope(s) located between amino acids 343 and 374.

Fine mapping of the T-cell epitopes with synthetic peptides. To further define the T-cell epitopes mapped by recombinant VV, overlapping peptides were synthesized. JK15, a DEN3 serotype-specific clone, recognized the region of NS3 between amino acids 234 and 283. DEN3 unique amino acids within this recognition region were used to predict the location of the minimal JK15 epitope. Amino acid sequences for the four serotypes of dengue virus were aligned between amino acids 234 and 283 and compared. As shown in Fig. 2A, two DEN3 unique amino acids (M at position 236 and T at position 248) were found within this 49-amino-acid sequence. We then tested peptides containing the second unique amino acid. Two peptides, 15a and 15b, which overlapped each other by five amino acids and which both contained this DEN3 unique amino acid were synthesized (Table 3). JK15 lysed target cells coated with peptide 15a but not with peptide 15b (Table 3), indicating that the epitope of JK15 is located between amino acids 239 and 253. The data obtained with both VV recombinants (Table 2) and peptides (Table 3) indicated that the JK15 epitope contained the unique amino acid at position 248 but not the one at position 236.

To further delineate the core epitope of JK15, an additional nine peptides, which were further deleted at either the C terminus or the N terminus, were synthesized. JK15 T cells lysed homologous target B cells sensitized with peptides 15c, 15d, 15e, 15g, and 15h but not targets sensitized with 15f, 15i, or 15j (Table 3). These results indicate that the sequence, IRYQTTATK, located between amino acids 241 and 249 is the minimal unit required for JK15 recognition.

JK44, a DEN1-, DEN2-, and DEN3-cross-reactive clone, recognizes the region between amino acids 194 and 214 on

TABLE 3. Analyses of the core epitopes recognized by JK15 and JK44 with synthetic peptides

Clone	Expt	Peptide	Amino acids in NS3	Amino acid sequence	% Specific ^{51}Cr release[a] at concn (μg/ml) of:		
					2.5×10^0	2.5×10^{-1}	2.5×10^{-2}
JK15	1	15a	239–253	LPIRYQTTATKSEHT	54	52	36
		15b	244–258	QTTATKSEHTGREIV	0	0	0
	2	15a	239–253	LPIRYQTTATKSEHT	59	52	—
		15c	239–251	LPIRYQTTATKSE	52	51	—
		15d	239–249	LPIRYQTTATK	32	7	—
		15e	241–253	IRYQTTATKSEHT	50	46	—
		15f	243–253	YQTTATKSEHT	10	2	1
	3	15a	239–253	LPIRYQTTATKSEHT	66	64	54
		15g	241–250	IRYQTTATKS	58	26	5
		15h	241–249	IRYQTTATK[b]	55	19	9
		15i	242–250	RYQTTATKS	9	2	2
		15j	242–249	RYQTTATK	5	3	3
JK44	1	44a	184–198	FKKRNLTIMDLHPGS	4	5	5
		44b	201–215	TRKYLPAIVREAIKR	49	12	3
	2	44b	201–215	TRKYLPAIVREAIKR	20	2	2
		44c	203–215	KYLPAIVREAIKR	5	1	1
		44d	205–215	LPAIVREAIKR	1	1	0
		44e	201–213	TRKYLPAIVREAI	23	15	2
		44f	201–211	TRKYLPAIVRE	37	28	6
	3	44f	201–211	TRKYLPAIVRE	30	26	15
		44g	201–210	TRKYLPAIVR	8	1	0
		44h	202–211	RKYLPAIVRE[b]	26	20	10
		44i	202–210	RKYLPAIVR	8	1	0

[a] Effector/target ratios for JK15 were 10 for experiment 1, 8 for experiment 2, and 12 for experiment 3; for JK44, effector/target ratios were 10 for experiments 1 and 3 and 12 for experiment 2. Specific lysis values for the negative control (no peptide) were 7% in experiment 1 and 0% in experiments 2 and 3 with JK15 and 6% in experiment 1 and 0% in experiments 2 and 3 with JK44. DEN3 antigen was also used in experiment 1 as a positive control, and the specific lysis values were 38% for JK15 and 71% for JK44. Dashes indicate experiments not done. Values representing significant levels of lysis are underlined.

[b] The smallest core sequence recognized by the indicated clone.

NS3. When the amino acid sequences for the four serotypes of dengue virus were aligned (Fig. 2B), three positions with amino acids that were common to DEN1, -2, and -3 but not DEN4 were found. Since JK44 recognized the D3-1 fragment but not the D3-10 fragment, two peptides were synthesized in this region. The 44a peptide contained amino acids 184 to 198, whereas the 44b peptide contained amino acids 201 to 215 and included three amino acids common to DEN1, DEN2, and DEN3, located at positions 204, 207, and 213. These peptides were tested in CTL assays. JK44 recognized peptide 44b but not peptide 44a (Table 3). To determine the minimum NS3 sequence required for JK44 recognition, we synthesized an additional seven peptides (44c, 44d, 44e, 44f, 44g, 44h, and 44i), which were further deleted at either the C terminus or the N terminus (Table 3). The JK44 clone lysed targets coated with peptide 44e, 44f, or 44h but did not lyse targets coated with peptide 44c, 44d, 44g, or 44i. Thus, the smallest peptide recognized by JK44 was determined to be the 10-amino-acid sequence, RKYLPAIVRE, located between amino acids 202 and 211 on the NS3 protein.

To define the JK13 and JK5 recognition regions located by the recombinant VV, a set of six 15-mer peptides, which overlapped each other by five amino acids and spanned the region from amino acids 333 to 372 (between D3-8 and D3-6) (Table 2), were synthesized and tested (Table 4). Both JK5 and JK13 lysed target cells coated with peptide 13-5d but did not lyse targets coated with the other five peptides (Table 4). Thus, the peptide 13-5d contains the epitope for both JK5 and JK13. These results are consistent with those obtained with recombinant VVD3-11. The protein expressed by D3-11 was truncated in the center of peptide 13-5d and was therefore not recognized by either JK13 or JK5 (Table 2). To determine the minimum sequence required for T-cell recognition by JK13 and JK5, six additional peptides (13-5g, 13-5h, 13-5i, 13-5j, 13-5k, and 13-5l), which were further deleted at either the C terminus or the N terminus, were synthesized (Table 4). JK13 lysed target cells coated with peptides 13-5g and 13-5i at a concentration of 2.5 μg/ml but did not lyse targets coated with peptide 13-5h, 13-5j, 13-5k, or 13-5l at the same concentration, indicating that the core epitope sequence recognized by JK13 is WITDFVGKTVW. However, at a higher peptide concentration (25 μg/ml), JK13 also lysed targets coated with peptides 13-5h and 13-5k (Table 4).

JK5 recognized peptide 13-5g at a concentration of 0.25 μg/ml but recognized peptide 13-5i only at a higher concentration (25 μg/ml). However, JK5 did not recognize peptides 13-5h, 13-5j, 13-5k, or 13-5l even at a concentration of 25 μg/ml (Table 4). The core epitope sequence recognized by JK5 is WITDFVGKTVW. Although the core sequence recognized by JK5 was identical to that recognized by JK13, JK5 recognized the core sequence only at concentrations that were 10 times higher than those required for recognition by JK13. JK13 also recognized peptides 13-5h and 13-5k at a concentration of 25 μg/ml, but JK5 did not. Both clones recognized the longer peptide (13-5d) at concentrations as low as 0.025 μg/ml.

A single DEN3 unique amino acid was identified at position 356 in NS3 (position 6 in the core sequence). However, no amino acid common to DEN1, DEN3, and WNV was found (Fig. 2C). With the exception of DEN1, all of the dengue virus strains used for the sequence comparisons were the same as those used to test the virus specificity of the T-cell clones. Because the published sequence of DEN1 (Singapore strain) (10) was used for the amino acid sequence comparison, but DEN1 (Hawaii strain) was used in the CTL assays, the epitope region of the DEN1 (Hawaii strain) was PCR amplified, cloned, and sequenced as described in Materials and Methods.

TABLE 4. Analyses of the core epitopes recognized by JK13 and JK5 with synthetic peptides

Expt	Peptide	Amino acids in NS3	Amino acid sequence	% Specific ^{51}Cr release[a] at concn (μg/ml) of:							
				2.5×10^{1}		2.5×10^{0}		2.5×10^{-1}		2.5×10^{-2}	
				JK13	JK5	JK13	JK5	JK13	JK5	JK13	JK5
1	13-5a	333–347	IQDEERDIPERSWNS	5	6	1	1	1	1	—	0
	13-5b	338–352	RDIPERSWNSGNEWI	0	4	1	2	2	0	—	2
	13-5c	343–357	RSWNSGNEWITDFVG	5	5	3	1	2	1	—	2
	13-5d	348–362	GNEWITDFVGKTVWF	66	84	66	78	46	64	34	51
	13-5e	353–367	TDFVGKTVWFVPSIK	14	15	4	0	0	0	—	0
	13-5f	358–372	KTVWTVPSIKAGNVI	8	12	1	0	0	0	0	0
2	13-5d	348–362	GNEWITDFVGKTVWF	—	—	83	79	82	74	71	63
	13-5g	350–362	EWITDFVGKTVWF	—	—	66	60	55	48	14	6
3	13-5g	350–362	EWITDFVGKTVWF	69	69	52	65	25	31	3	2
	13-5h	348–360	GNEWITDFVGKTV	54	10	19	1	0	0	0	0
	13-5i	351–361	WITDFVGKTVW[b]	62	57	41	1	10	0	0	0
	13-5j	352–361	ITDFVGKTVW	11	2	1	1	0	0	0	0
	13-5k	351–360	WITDFVGKTV	38	1	5	0	0	0	0	0
	13-5l	352–360	ITDFVGKTV	0	0	0	0	0	0	0	0

[a] Effector/target ratios were 8 for experiments 1 and 3 and 9 for experiment 2. DEN3 antigen was used in experiment 1 as a positive control, and the specific lysis values were 47% for JK13 and 48% for JK5. Specific lysis values for the negative control were 0% in experiments 1 and 3 and 2% in experiment 2 with JK13 and 0% in experiments 1 and 3 and 3% in experiment 2 with JK5. Dashes indicate experiments not done. Values representing significant levels of lysis are underlined.

[b] Smallest core sequence recognized by JK13 and JK5.

The sequence of the DEN1 (Hawaii strain) between amino acids 348 and 365 was identical to that of DEN1 (Singapore strain).

To confirm that JK5 recognizes the core sequences of the dengue virus serotypes with which it cross-reacts and to determine which amino acid residues are critical for recognition by JK5 and JK13, an additional six 11-mer peptides (13-5i-D1, 13-5i-D2, 13-5i-D2K, 13-5i-D4, 13-5i-D4Q, and 13-5i-WNV) were synthesized. 13-5i-D1, 13-5i-D2, 13-5i-D4, and 13-5i-WNV represent the core sequences for DEN1, DEN2, DEN4, and WNV, respectively. 13-5i-D2K and 13-5i-D4Q were mutated at positions 352 and 355, so that they matched the sequence in DEN3 except at position 356 (Fig. 3). Consistent with its serotype specificity, JK13 recognized 13-5i but not any of the other six peptides. JK5 recognized peptides 13-5i, 13-5i-D1, and 13-5-WNV but not 13-5i-D2, 13-5i-D2K, 13-5i-D4, or 13-5i-D4Q (Fig. 3). Thus, JK5 recognizes the core sequences of each of the viruses with which it cross-reacts, whereas JK13, a DEN3-specific clone, recognizes only the DEN3 core sequence. The amino acid at position 356 (position 6 in the core epitope) is critical for recognition, since amino acid substitutions at that position (V to K in DEN2 and V to Q in DEN4) abolished recognition. The peptide concentration required for JK5 recognition of the DEN1 epitope was similar to that required for recognition of the DEN3 core sequence (Table 4). However, a fourfold-higher concentration of peptide 13-5-WNV than of peptide 13-5i was required for recognition by JK5 (data not shown), suggesting that JK5 has a lower affinity for the WNV sequence.

		JK13	JK5
13-5i	WITDFVGKTVW	+	+
13-5i-D1	-----P-----	-	+
13-5i-D2	-V---K-----	-	-
13-5i-D2K	-----K-----	-	-
13-5i-D4	----YQ-----	-	-
13-5i-D4Q	-----Q-----	-	-
13-5i-WNV	---EYI-----	-	+/-
13-5d	GNEWITDFVGKTVWF	+	
13-5d-D1	-Y------P-----	-	
13-5d-D2	-H--V---K-----	-	
13-5d-D2K	--------K-----	-	
13-5d-D2V	----V---------	+	
13-5d-D4	-FD----YQ-----	-	
13-5d-D4Y	-------Y------	-	
13-5d-D4Q	--------Q-----	-	

FIG. 3. Analysis of the cytotoxic activities of single residue substitutions within the JK13/JK5 epitope. The sequences of the peptides 13-5i, 13-5i-D1, 13-5i-D2, 13-5i-D4, 13-5i-WNV, 13-5d, 13-5d-D1, 13-5d-D2, and 13-5d-D4 corresponded to those present in each serotype of dengue virus and WNV. Peptides 13-5i-D2K, 13-5i-D4Q, 13-5d-D2K, 13-5d-D2V, 13-5d-D4Y, and 13-5d-D4Q contained a single amino acid substitution compared with the sequence in DEN3. The results from T-cell cytotoxic assays are indicated as positive or negative. +/− indicates that although JK5 did not recognize peptide 13-5i-WNV at the same concentration as the other peptides, it did recognize this peptide at a fourfold-higher concentration. The conditions used for the CTL assay are the same as those described in Table 4, footnote *a*.

Within the core sequence for JK13, there is a single substitution (V to P) at position 356 (position 6 in the core sequence) in the DEN1 sequence. This mutation is sufficient to abolish JK13 recognition. Two substitutions are present within the core sequences of DEN2 and DEN4. To determine which amino acid substitution abolished JK13 recognition, additional peptides were synthesized. Since the 13-mer peptide 13-5g and the 15-mer peptide 13-5d showed higher activity than the 11-mer core sequence with both the JK13 and JK15 clones (Table 4), the 15-mer peptide was used in this study to ensure good levels of activity. Peptides 13-5d-D1, 13-5d-D2, and 13-5d-D4 contained the core sequences for DEN1, DEN2, and DEN4, respectively. Peptides 13-5d-D2K, 13-5d-D2V, 13-5d-D4Y, and 13-5d-D4Q all contained a substitution in the N-terminal flanking region from H to N at position 349 in DEN2 and from FD to NE at positions 349 and 350 in DEN4, and thus this region was identical to the DEN3 sequence. Each of these four peptides also contained a single substitution within the core

TABLE 5. Determination of HLA class II restriction of JK15, JK13, JK5, and JK44

Target	HLA class II type[a]			% Specific ^{51}Cr release[b]			
	DR	DP	DQ	JK15	JK13	JK5	JK44
Autologous	15 (=2), 103	W2	W5 (=W1), W6 (=W1)	57	46	74	43
3107	**2**	W4	W2	64	47	63	23
3161	**2**	—	W2	48	27	62	—
9038	12	**W2**	W7	0	6	7	5
MS	1,4	W4	**W1**, W3	0	8	7	4
VA-9	**15**, 17	—	W2, **W6**	44	52	32	15

[a] Known HLA loci matching that of the T-cell donor are in boldface. —, locus for which HLA type is not known.
[b] Effector/target ratios were 10 for JK15 and JK5 and 5 for JK44 and JK13. Peptide 15a (0.25 μg/ml) and peptide 44b (2.5 μg/ml) were used to coat target cells for lysis by JK15 and JK44, respectively. Peptide 13-5d (0.25 μg/ml) was used to coat target cells for lysis by both JK13 and JK5. The CTL assay time was 4 h. Values representing significant levels of lysis are underlined. The dash indicates that the experiment was not done.

sequence (Fig. 3), and thus each differed by only one amino acid from the DEN3 sequence. JK13 recognized peptides 13-5d and 13-5d-D2V but not peptides 13-5d-D2, 13-5d-D2K, 13-5d-D4, 13-5d-D4Y, and 13-5d-D4Q. Thus, failure of JK13 to recognize the DEN2 core sequence was observed after the substitution of V with K at position 6 in the peptide but not after the conservative substitution of I with V at position 2. Either of the substitutions in DEN4, at position 5 or 6, was sufficient to abolish recognition by JK13. Thus, the amino acids in both positions 5 and 6 are critical for JK13 recognition. Unfortunately, the stock of JK5 cells was depleted prior to the testing of this second set of peptides with a single amino acid substitution.

HLA restriction patterns of the T-cell clones. The HLA restriction pattern for JK44 was analyzed by using anti-HLA-DR, anti-HLA-DP, and anti-HLA-DQ MAbs. These MAbs inhibit the cytotoxic activity of homologous HLA-restricted T cells in a CTL assay. Only the HLA-DR MAb inhibited lysis of target cells by JK44, indicating that JK44 is HLA-DR restricted (data not shown).

To determine the HLA-DR allele restriction element for JK44 and the restriction patterns for the other three clones, allogeneic target cells, 3107, 3161, 9038, MS, and VA-9, were used in CTL assays. The HLA subtype pattern for each of these target cells was defined (Table 5). Allogeneic target cells 3107, 3161, and VA-9 share HLA-DR15 (DR2) and present the appropriate T-cell specific peptides, 15a and 13-5d, for recognition by JK15 and JK13/JK5 T-cell clones, respectively. Target cells 3107 and VA-9 can also present peptide 44b for recognition by JK44 (Table 5). Cell lines 9038 and MS, which share HLA-DPw2 and HLA-DQw1 subtypes with the autologous target cells, respectively, could not present epitopes recognized by JK15, JK13, JK5, or JK44 (Table 5). The results indicate that JK15, JK13, JK5, and JK44 are HLA-DR15 restricted.

Determination of the TCR Vβ chain usage for JK13 and JK5. To determine whether JK13 and JK5 arose from the same lineage, the Vβ chains of each clone were sequenced. Reverse transcription-PCR was performed on RNA extracted from JK13 and JK15 T cells by using a specific 3′ primer, which is located in the TCR β-chain constant region, and 1 of 26 5′ primers located in the variable region of the TCR β chain. Of the 26 PCRs performed for JK5, only the one in which a 5′ specific primer for the Vβ1 variable region was used yielded a PCR product of the expected size, indicating Vβ1 usage by the TCR of JK5. Only the PCR using the 5′ primer specific for the Vβ8.1 variable region produced an appropriate-size PCR product for JK13, indicating that Vβ8.1 is used by the TCR of JK13.

To obtain the amino acid sequences of the β chains of the JK5 and JK13 TCRs, the PCR products were sequenced. Figure 4 shows the junction sequences located between the variable region and the constant region of the β chains for the JK13 and JK5 TCRs. Although these two sequences are highly conserved, some divergence was observed within the NDN region and at the beginning of the Jβ1.2 region. These results indicate that JK13 and JK5 are not from the same lineage and that two CTL clones with different β chains in their TCRs can recognize the same epitope.

DISCUSSION

We have characterized four human CD4$^+$ T-cell clones derived from a donor who experienced dengue illness following receipt of a live experimental DEN3 vaccine. Core epitopes for these clones map to three different sequences, 241-IRYQT-TATK-249 for a DEN3-specific clone, JK15; 202-RKY-LPAIVRE-211 for a DEN1-, DEN2-, and DEN3-cross-reactive clone, JK44; and 351-WITDFVGKTVW-361 for both a DEN3-specific clone, JK13, and a DEN1-, DEN3-, and WNV-cross-reactive clone, JK5. Although the JK13 and JK5 clones recognized the same core epitope in DEN3, they varied in virus specificity and in the efficiency with which they recognized targets coated with a small peptide. Sequence analysis of the variable regions of the TCRs of the JK13 and JK5 clones indicated that these two clones use different β chains. These studies indicate that a single peptide can induce the response of T cells that possess different TCRs. The various TCRs may interact with the same peptide in different ways, and this interaction may play a role in determining the serotype specificity of the clones. In support of this hypothesis, the serotype-specific T-cell clone, JK13, required a lower concentration of core peptide than did the serotype cross-reactive T-cell clone, JK5. Similarly, several CD8$^+$ T-cell clones induced by the lymphocytic choriomeningitis virus NP protein in *H-2*^{d} mice recognized the same short peptide sequence, even though these T-cell clones differed slightly from each other in serotype specificity and in affinity for this minimal epitope (38). Also, dengue virus-specific, HLA-B35-restricted, peptide-induced CD8$^+$ human T cells had different affinities for the inducing

	Vβ 1	NDN	Jβ1.2	Cβ
JK5	CASS	VGD	DYGYTFGSGTRLTVV	ED
JK13	CASSL	VGHRAY	FGSGTRLTVV	ED
	Vβ 8.1	NDN	Jβ1.2	Cβ

FIG. 4. Sequence comparison of JK13 and JK5 TCR Vβ chains. The JK13 and JK5 TCR Vβ chain regions were PCR amplified and sequenced. The sequences of the V-D-J region and the constant region (C) of each TCR are shown.

peptide and were heterogeneous in their virus serotype specificities (27, 39).

The existence of four serotypes of dengue virus provides a unique opportunity to study how T-cell clones discriminate some naturally occurring sequences but not others. With this system, it is possible to analyze both the positions and the particular amino acids within an epitope that are important for T-cell recognition. Unlike most other viral immunodominant proteins which contain one or two T-cell epitopes (6, 15, 37), our studies (26) have shown that the dengue virus NS3 protein contains at least four epitopes for this donor. The core epitope in the DEN3 NS3 sequence recognized by clone JK15 differed from the one not recognized in the DEN1 sequence by a single substitution (T to V) at position 8 (Fig. 2A). JK15 also did not recognize the DEN2 or DEN4 sequence. The DEN4 sequence contained two substitutions, the same T-to-V change at position 8 as in the DEN1 sequence and an additional substitution, from T to P, at position 6. The DEN2 sequence contained three substitutions in this region, T to P at position 6, T to I at position 8, and K to R at position 9. The amino acid substitution at position 9 of the DEN2 sequence is conservative, but the substitutions at positions 6 and 8 are not. This result suggests that the amino acid at position 8 is critical for recognition by JK15. In addition, the amino acid at position 6 and possibly also the one at position 9 may play a role in recognition.

The JK44 clone can tolerate two conservative amino acid substitutions, one at position 1 in DEN2 (R to K) and one at position 2 in DEN1 and DEN2 (K to R) (Fig. 2B). However, two additional substitutions in DEN4, Y to I at position 3 and A to S at position 6, were not tolerated, indicating that the amino acid in either or both of these positions is important for T-cell recognition.

Although JK13 and JK5 recognize the same minimal core sequence in DEN3, JK5 also recognizes the core sequences of DEN1 and WNV. Since this epitope is presented by the same HLA-DR15 molecule to both clones, the amino acid variations within the core sequence among the DEN1, DEN3, and WNV sequences would not be expected to be important for HLA recognition. The substitution of P for V at position 6 of the core sequence generated a peptide which was not recognized by JK13 but was recognized by JK5, indicating that the amino acid at position 6 is involved in the epitope-TCR interaction. Substitution of K for V or Q for V at position 6 of the core sequence was not tolerated by either JK13 or JK5, and substitution of Y for F at position 5 was not tolerated by JK13, indicating that while position 6 is critical for both JK13 and JK5 recognition, position 5 may also be important for JK13 recognition. The interaction of the TCR with a peptide bound to an HLA molecule determines the serotype specificity of CD4$^+$ CTL clones. The amino acids within the peptide that are critical for HLA-DR15 binding have not been well defined, as discussed below. The data obtained with the JK13 and JK5 clones suggest that the site on the TCR that reacts with the HLA-bound peptide is less restrictive in the JK5 clone than in the JK13 clone. None of the peptides with substitutions at position 6 could be recognized by JK13, but JK5 could recognize peptides in which position 6 was substituted by another nonpolar hydrophobic amino acid (Fig. 3). This would explain the unusual cross-reactivity pattern of the JK5 clone. The amino acid at position 6 of this epitope may be critical for recognition by the CTL clones, or alternatively, it may indirectly affect CTL binding by altering the way in which the critical residues for recognition are exposed on the HLA-DR15 molecule (37).

Since the amino acid sequence homology among the four serotypes of dengue virus is fairly high (67 to 75%) (10), one would expect the epitopes of serotype-cross-reactive clones to be located in regions of highly conserved sequence and, if they are not, that these clones would be more tolerant of substitutions than the serotype-specific clones. A previously described core epitope, 255-EIVDLMCHAT-264, for the HLA-DPw2-restricted, four-serotype-cross-reactive clone JK34 mapped to a completely conserved region on the dengue virus NS3 protein. However, a conservative substitution of V for L at position 5 of this core sequence in WNV abolished T-cell recognition (20). On the other hand, two HLA-DR15 restricted clones, JK43 and JK4, which were four serotype and flavivirus cross-reactive and recognized the same core sequence on NS3 (26), as well as JK44, the three-serotype-cross-reactive clone defined in this study (Fig. 2), could tolerate conservative amino acid substitutions in the first two positions of their core sequences. Sequence recognition by serotype-specific T cells would be expected to be very restricted. However, the DEN3-specific clone, JK13, tolerated a conservative amino acid substitution from I to V at position 2 of the core sequence (Fig. 3). Similarly, the first two amino acids of the core sequence of an HLA-DR2-restricted T-cell epitope from *Mycobacterium leprae* could be replaced by almost any other amino acid and still be recognized (1). These results suggest that the core sequences recognized by both HLA-DR15-restricted, dengue virus serotype-specific and serotype-cross-reactive clones do not differ markedly in their sequence conservation among the four dengue virus serotypes. Also, both serotype-specific and serotype-cross-reactive clones could tolerate conservative amino acid substitutions at one of the first two positions. The serotype cross-reactive T-cell clone, JK5, could also tolerate some but not all amino acid substitutions at positions 4 (E to D), 5 (Y to F), and 6 (P or I to V) (Fig. 3).

Naturally processed class II peptides usually contain 13 to 25 amino acids (8, 34). Studies of these peptides after deletion of amino acids from both the C and N termini have shown that the minimal length of an active class II peptide is about 9 to 10 amino acids (17). The lengths of the core epitopes for JK15, JK13/JK5, and JK44 were determined to be 9, 11, and 10 amino acids, respectively. The core sequences for two additional dengue virus-cross-reactive clones, JK43/JK4 and JK34, were also shown to be 9 and 10 amino acids in length, respectively (20, 26). These results are consistent with other studies of T-cell class II epitopes in human viruses, in which core sequences of CD4$^+$ epitopes from hepatitis B virus and human immunodeficiency virus type 1 were found to be 10 and 12 amino acids in length, respectively (6, 15). Studies with HLA-eluted peptides have shown that deletion of two amino acids from either the N terminus or the C terminus of an 18-mer or 17-mer peptide did not decrease the peptide's ability to bind to class II molecules, but further deletions did lead to some reduction in binding, although activity could still be demonstrated for peptides as short as nine amino acids (17). In our study, the longer 15-mer peptides, such as 15a and 13-5d, induced a higher level of lysis than did the shorter core sequences, 15h and 13-5i, and the T-cell clones required 100- to 1,000-fold less of a 15-mer peptide than of the shorter core sequences for recognition. This finding suggests that the flanking sequences assist in the presentation of the peptide.

The four T-cell clones used in this study are HLA-DR15 restricted. HLA-DR2 was recently reclassified as HLA-DR15 and HLA-DR16 (3). Two consensus binding motifs for HLA-DR2 were previously proposed on the basis of the sequences of endogenous peptides eluted from HLA-DR2a/DR2b molecules (Fig. 5). An i + 10th motif was observed in 7 of 10 peptides, where i represents I, L, or V and + 10 indicates that a positively charged amino acid (H, K, or R) is present at

JK15	241-IRYQTTAT**K**-249
JK44	202-RKYLPAIV**R**E-211
JK13 and JK5	351-WITDFVG**K**TVW-361
JK43 and JK4	146-VIGLYGNGV-154
M.leprae-specific	LQAAPALD**K**L
HLA-DR2a/DR2b	iXXXXXXXXX+
HLA-DRβ*1502	VYXXX+

FIG. 5. Comparison of the dengue virus NS3 core epitopes with other HLA-DR2-restricted epitopes. Numbers beside the amino acid sequences represent the peptide positions in DEN3 NS3. Boldface amino acids represent conserved positively charged residues. The epitope for *M. leprae* is from reference 1; the motif for HLA-DR2a/DR2b is from reference 8; the motif for HLA-DRβ*1502 is from reference 16.

position 10 (8). In contrast, another study proposed a VK + 4 motif for HLA-DR2 (genotype DRβ1*1502) peptides (16). Because naturally processed, HLA class II-restricted peptides usually contain more amino acids than are required for HLA binding and T-cell recognition, motifs predicted on the basis of amino acid conservation in eluted peptides may not accurately predict the region of the epitopes critical for HLA binding since the minimal epitope region within these peptides cannot be identified. The DR15-restricted dengue virus NS3 minimal epitope sequences that we defined in this study as well as an additional DR15-restricted dengue virus NS3 epitope, 146-VIGLYGNGV-154, reported previously (26) do not appear to fit either of the two proposed motifs (Fig. 5). When the core sequence for JK15 (Fig. 2A) is extended two residues at the N-terminal end, it does fit the $i + 10$th motif (i = L and K at position 10), but these two additional amino acids (L and P) do not appear to be essential for the binding of this peptide to the HLA molecule (Table 3, peptides 15a and 15e). These data suggest that an L in the i position may not be important for peptide recognition. Comparison of the minimal recognition sequences defined on the dengue virus NS3 protein indicates that a positive amino acid, K or R, is located at position 8 or 9 (Fig. 5). A DR2-restricted epitope, LQAAPALDKL, previously defined for *M. leprae* also contains a K in position 9. Although the first two amino acids or the last amino acid in the *M. leprae* core sequence could be replaced with almost any other amino acid without affecting recognition by T cells, the other amino acids, including K at position 9, were essential for recognition (1). Since the majority of the peptides that bind to the HLA-DR2 molecule have a positively charged amino acid at or near the C terminus of the minimal epitope sequence, it is likely that this residue is involved in the interaction with the HLA-DR2 molecule. However, since HLA-DR2 has now been subdivided into HLA-DR15 and HLA-DR16, and within the HLA-DR2 population HLA-DR15 is the minority isotype (8), further studies are required to define and compare the motifs critical for HLA-DR15 and HLA-DR16 molecule binding.

This study provides additional data about the human T-cell response to dengue virus infection that will be important for future analyses of T-cell responses in patients with DHF. A precise understanding of human immune responses to dengue virus is also necessary as an aid to the development of a safe and effective vaccine which can simultaneously induce long-lasting protective immunity to the four serotypes of dengue viruses.

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