Definition of the region on NS3 which contains multiple epitopes recognized by dengue virus serotype-cross-reactive and flavivirus-cross-reactive, HLA-DPw2-restricted CD4+ T cell clones

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The epitopes recognized by six CD4+ CD8+ cytotoxic T lymphocyte (CTL) clones established from a dengue-3 virus-immune donor were defined. (i) Three CTL clones, JK10, JK34 and JK39, were cross-reactive for dengue virus types 1–4. (ii) One clone, JK28, was cross-reactive for dengue virus types 1–4 and West Nile virus. (iii) Two clones, JK26 and JK49, were cross-reactive for dengue virus types 1–4, West Nile virus and yellow fever virus. The clones, except for JK49, recognized the same epitope on NS3 in an HLA-DPw2-restricted fashion. The smallest synthetic peptide recognized by the five CTL clones was a 10 aa peptide which comprises aa 255–264 on dengue virus NS3. JK49 recognized the overlapping epitope which comprises aa 257–266 in an HLA-DPw2-restricted fashion. Analysis of T cell receptor (TCR) usage by these T cell clones revealed that (i) JK10 and JK34 use Vα11, and JK34 and JK28 use Vβ23, and (ii) the amino acid sequences of the V(D)J junctional region of the TCR were different among these five CTL clones. There were, however, single amino acid conservations among TCRs of some of these T cell clones. These results indicate that the region on NS3 which comprises aa 255–266 contains multiple epitopes recognized by dengue serotype-cross-reactive and flavivirus-cross-reactive CD4+ CTL in an HLA-DPw2-restricted fashion and that a single epitope can be recognized by T cells which have heterogeneous virus specificities.

Introduction

The Flaviviridae includes at least 66 viruses that are transmitted by mosquitoes, ticks and unknown vectors (Monath, 1990). Dengue viruses are members of the family Flaviviridae and are transmitted to humans mainly by Aedes aegypti mosquitoes (Monath, 1994). Four serotypes of dengue virus exist, types 1, 2, 3 and 4. Infection with any of the four serotypes of dengue virus can result in an asymptomatic infection, a self-limited febrile disease (dengue fever) or a severe, life-threatening illness (dengue haemorrhagic fever) (Halstead, 1988). The pathogenesis of dengue haemorrhagic fever is not understood.

Primary infection with one serotype of dengue virus induces virus-specific CD4+ and CD8+ memory T lymphocytes (Kurane & Ennis, 1994). These include (i) serotype-specific, (ii) dengue serotype-cross-reactive and (iii) flavivirus-cross-reactive T lymphocytes. The patterns of serotype-cross-reactivities are heterogeneous. We have previously reported CD4+ T lymphocytes which are cross-reactive for all the four serotypes (Kurane et al., 1991), cross-reactive for dengue-1, dengue-2 and dengue-3 (Kurane et al., 1991; Zeng et al., 1996), cross-reactive for dengue-2, dengue-3 and dengue-4 (Kurane et al., 1991), cross-reactive for dengue-1, dengue-3 and dengue-4 (Kurane et al., 1991), cross-reactive for dengue-2 and dengue-4 (Livingston et al., 1995), and cross-reactive for dengue-1 and dengue-3 (Green et al., 1993). We have also reported CD8+ cytotoxic T cell clones cross-reactive for dengue-2 and dengue-4 (Livingston et al., 1995).

It is generally accepted that the virus-specificities of T lymphocytes are mainly based on the degree of conservation in the amino acid sequence of the epitopes. Thus, T cells which recognize epitopes that are not conserved among viruses are specific for that virus, while T cells which recognize epitopes that are conserved among viruses are virus-cross-reactive.
However, this is not always the case. We have reported that two groups of dengue virus-specific CD8+ CTL clones which include dengue-4-specific CD8+ CTL clones and dengue-2, dengue-4-cross-reactive CTL clones recognize the same epitope (aa 500–508 on NS3) in an HLA-B35-restricted manner (Livingston et al., 1995). This result suggests that virus specificity of T lymphocytes is not solely determined by the degree of amino acid conservation in the epitopes among viruses.

In this paper we report that five CD4+ T cell clones which are dengue serotype-cross-reactive or flavivirus-cross-reactive recognize the same minimum epitope on NS3 in an HLA-DPw2-restricted fashion. Furthermore, one flavivirus-cross-reactive CD4+ T cellclone recognizes the epitope which is shifted towards the C terminus by two amino acids. It is likely that these dengue serotype-cross-reactive and flavivirus-cross-reactive T lymphocytes are activated by a secondary infection with a serotype different from that which caused the primary infection. These cross-reactive T lymphocytes probably play an important role in immune responses by providing help for antibody production and by lysing virus-infected cells. Presence of the region on NS3 which includes epitopes recognized by CD4+ T cells of multiple virus specificities suggests that development of dengue serotype and flavivirus-cross-reactive subunit vaccines may be possible.

Methods

**Human peripheral blood mononuclear cells (PBMC).** A peripheral blood specimen was obtained from a healthy adult who had been immunized with yellow fever vaccine 6 months earlier and was immunized with an experimental dengue-3 virus (strain CH53489) vaccine 1 year previously (Innis et al., 1988a). The PBMC were separated using a Ficoll–Hypaque density gradient centrifugation method. PBMC were resuspended at a concentration of 10⁷/ml in RPMI 1640 containing 10% FCS (Gibco-BRL Life Technologies) and 10% DMSO (Fisher Scientific) and were cryopreserved until use. The HLA class II alleles of the donor were DRB1*0103, DRB1*0501, DRB5*0101, DQB1*0501, DQA1*01, DPB1*0201, which is serologically defined as HLA-DR103, DR15, DQ5, DQ6, DPw2 (Bodmer et al., 1994).

**Preparation of virus antigen.** Dengue virus type 1, Hawaii strain; type 2, New Guinea C strain; type 3, CH53489 strain; and type 4, 814669 strain; yellow fever virus (YFV), 17D strain; and West Nile virus (WNV), E101 strain were used. Virus antigens were prepared as previously reported (Kurane et al., 1993). After incubation at 37 °C for 5 h, the supernatant was collected from each well and counted in a automated gamma counter (Packard). The percent specific ⁵¹Cr release was calculated by the formula: (c.p.m. experimental release − c.p.m. spontaneous release) x 100/c.p.m. maximal release − c.p.m. spontaneous release).

**RNA collection and complementary DNA (cDNA) synthesis.** Total RNA was collected by acid guanidinium– phenol–chloroform method (Chomczynski & Sacchi, 1987). Briefly, 5 x 10⁶ cells were pelleted in a 1.5 ml Eppendorf microcentrifuge tube after washing with PBS and stored at −70 °C until use. After treating with denaturing solution, RNA was extracted with phenol–chloroform under acidic conditions. The inorganic phase was transferred to a new Eppendorf tube and precipitated with isopropanol twice. The RNA precipitate was re-dissolved and treated with DNase I, then re-precipitated with ethanol. The HLA class II alleles of the donor were DRB1*0103, DRB1*0501, DRB5*0101, DQB1*0501, DQA1*01, DPB1*0201, which is serologically defined as HLA-DR103, DR15, DQ5, DQ6, DPw2 (Bodmer et al., 1994).

**Establishment of dengue virus-specific T cell clones.** Dengue virus-specific CD4+ CTL clones were established from PBMC using a limiting dilution method as previously reported (Kurane et al., 1991). PBMC (4 x 10⁵) were cultured with dengue-3 virus antigen at a final dilution of 1:30 in 0.2 ml RPMI 1640 containing 10% human AB blood type serum in 96-well round-bottom plates (Costar) for 7 days. On day 7, blastoid cells were enriched by Ficoll–Hypaque density gradient centrifugation and were cultured at concentrations of 30, 10, and 3 cells per well with γ-irradiated (3000 rads) 1 x 10⁶ autologous PBMC in 0.2 ml RPMI 1640 containing 10% human AB serum, 10% TCGF (Cellular Products), and dengue-3 virus antigen at a final dilution of 1:30 in 96-well round-bottom plates. On day 14, 0.1 ml of medium was removed from each well and 1 x 10⁶ γ-irradiated autologous PBMC in 0.1 ml of fresh medium with human AB serum, 10% TCGF and dengue-3 virus antigen was added to maintain the same final concentration described above. On day 21, cells in wells demonstrating growth were transferred to 48-well flat-bottom plates (Costar) and were further cultured with 1 x 10⁶ γ-irradiated autologous PBMC in 1 ml RPMI 1640 containing 10% human AB serum, 10% TCGF and dengue-3 virus antigen.

**Peptide synthesis.** Peptides were synthesized according to the published amino acid sequence of the dengue-4 gene (Mackow et al., 1987) using the RAMPs multiple peptide synthesis system (New England Nuclear) as previously reported (Takahashi et al., 1991). Peptides were also synthesized using the Symphony peptide synthesizer (Rainin Instruments) at the University of Massachusetts Peptide Core Facility. Amino acid analyses to verify amino acid composition of peptides were performed by Dr Robert Carraway at the University of Massachusetts Medical Center Peptide Core Facility.

**Cytotoxic assays.** Target cells (0.5–2 x 10⁶) were labelled with 0.5 mCi Na⁹⁵CrO₄ (New England Nuclear) in 0.2 ml RPMI 1640 containing 10% FCS at 37 °C for 30 min. Labelled cells were washed three times and resuspended at 1 x 10⁶/ml in RPMI 1640 containing 10% FCS; 0.1 ml of this cell suspension was added to each well in V-bottom microtitre plates (Linbro Chemical). Various concentrations of effector cells in 0.1 ml RPMI 1640 containing 10% FCS were added to each well to give the described effector:target ratios. In cytototoxic assays using peptides, 1 x 10⁶ target cells in 0.1 ml were incubated with peptide in 0.05 ml for 30 min, and effector cells in 0.05 ml were added to each well (Kurane et al., 1993). After incubation at 37 °C for 5 h, the supernatant was collected from each well and counted in an automated gamma counter (Packard). The percent specific ⁵¹Cr release was calculated by the formula: (c.p.m. experimental release − c.p.m. spontaneous release) x 100/c.p.m. maximal release − c.p.m. spontaneous release).

**T cell receptor (TCR) mRNA detection by symmetric PCR.** Synthesized total cDNA was diluted to 700 µl with 1/20 Tris–EDTA (pH 7.5) and 10 µl of this solution was used in each PCR reaction. TCR Vα- and Vβ-specific 3′ primers, TCR Cα- and Cβ-specific 3′ primers and β-actin-specific 3′ and 3′ primers were synthesized as previously reported (Okamoto et al., 1994). The reaction mixture was first denatured at 95 °C.
for 5 min and then kept at 85 °C. During this incubation Taq DNA polymerase and MgCl₂ were added for a hot start (Nuovo et al., 1991). To amplify TCR α chain cDNA, 31 cycles of PCR were done with the cycle of 95 °C for 1 min (1.5 min in the first cycle), 46 °C for 1.5 min and 72 °C for 1 min. To amplify TCR β chain cDNA, 26 cycles of PCR were performed with the cycle of 95 °C for 1 min (1.5 min in the first cycle), 55 °C for 1 min and 72 °C for 1 min. β-actin message was amplified in each reaction simultaneously as an internal control. Following the PCR, the total reaction mixture was electrophoresed on a 5% polyacrylamide gel.

**Direct sequencing of the PCR-amplified TCR message.** We first determined Vα and Vβ usage as described above, and then amplified the TCR Vα and Vβ mRNA by RT–PCR with primers specific for the determined V gene. Total PCR product was separated on 5% low melting point agarose gel and the amplified fragment was purified using the GeneClean kit (Bio 101). ssDNA was synthesized on both strands by the asymmetric PCR method, and used for direct sequencing (Innis et al., 1988b). Sequencing reactions were primed with the 32P-end-labelled primers specific for the determined V gene, 3’ Cα-specific internal primer or 3’ Cβ-specific internal primer (Okamoto et al., 1994). Reactions were performed at 70 °C for 15 min with sequence grade Taq polymerase (Promega) and stopped by adding formamide stopping dye. These reactions were electrophoresed on a 6% polyacrylamide–urea gel and autoradiographed after drying.

**Amplification of the TCR message by the SLIC-RACE method.** When the TCR V gene usage could not be detected by symmetric PCR using the 25 Vα-specific and 26 Vβ-specific primer sets, the TCR was amplified by the SLIC-RACE method using a 5’-AmpliFinder RACE kit (Clontech). Total cellular RNA was collected from 5 × 10⁶ live cells as described above. After purifying the polyA⁺ RNA by the PolyATtract mRNA isolation system (Promega), cDNA was synthesized using A-MuLV RT at 52 °C primed with the Cα-specific oligonucleotide (5’ ATCTAATAATTCGGTACAGTTCC 3’). After hydrolyzing RNA, cDNA was purified by GENO-BIND following the manufacturer’s protocol and precipitated by ethanol. This precipitate was re-dissolved in water and used for anchor ligation. Anchor-ligated cDNA was amplified by PCR using 5’-AmpliFinder anchor primer supplied by the manufacturer and a 3’ TCR Cα-specific primer (5’ CTTGTCACGAGTTAGAGTCCTC 3’). Thirty-seven cycles of PCR were done with denaturing at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. The PCR product was separated on a 15% low melting point agarose gel and purified using a GeneClean kit. The strands were sequenced using a dsDNA Sequencing System (Gibco-BRL Life Technologies). Primers used for sequencing were the 5’-AmpliFinder anchor primer and the internal Cα-specific primer (5’ GGTACACGGCAGGTCAGGGTTC 3’).

**Results**

**Virus specificities and protein recognition of the CD4⁺ CTL clones**

Six CD4⁺ CD8⁻ CTL clones (JK10, JK26, JK28, JK34, JK39 and JK49) were established from T cells of a donor who had been immunized with experimental dengue-3 vaccine, using a limiting dilution method as previously described (Kurane et al., 1989b, 1991). JK10, JK34 and JK39 were cross-reactive for dengue virus types 1–4, JK28 was cross-reactive for dengue virus types 1–4 and WNV, and JK26 and JK49 were cross-reactive for dengue virus types 1–4, WNV, and YFV (Table 1). We determined that all of these clones recognized NS3 protein using purified dengue proteins or recombinant vaccinia viruses which expressed dengue virus proteins (data not presented).

**Definition of the peptides recognized by CD4⁺ CTL clones**

We demonstrated that the epitopes recognized by these six CTL clones were located between aa 247 and 340 in CTL clones (Table 2). JK26 and JK28 recognized only peptide reactive for dengue virus types 1–4, WNV and YFV (Table 1). We determined that all of these clones recognized NS3 protein using purified dengue proteins or recombinant vaccinia viruses which expressed dengue virus proteins (data not presented).

**Table 1. Dengue virus-specific CD4⁺ CD8⁻ CTL clones used in the present paper**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Dengue serotypes and flavivirus specificities</th>
<th>Protein recognition</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK10</td>
<td>D1, D2, D3, D4</td>
<td>NS3</td>
</tr>
<tr>
<td>JK34</td>
<td>D1, D2, D3, D4</td>
<td>NS3</td>
</tr>
<tr>
<td>JK39</td>
<td>D1, D2, D3, D4</td>
<td>NS3</td>
</tr>
<tr>
<td>JK28</td>
<td>D1, D2, D3, D4, WNV</td>
<td>NS3</td>
</tr>
<tr>
<td>JK26</td>
<td>D1, D2, D3, D4, WNV</td>
<td>NS3</td>
</tr>
<tr>
<td>JK49</td>
<td>D1, D2, D3, D4, WNV</td>
<td>NS3</td>
</tr>
</tbody>
</table>

* D1, Dengue-1 virus; D2, dengue-2 virus; D3, dengue-3 virus; D4, dengue-4 virus; WNV, West Nile virus; YFV, yellow fever virus.

**Table 2. Definition of the peptides recognized by CD4⁺ CTL clones**

Effector:target ratios were 5:1 for JK10, 6:1 for JK49 and 10:1 for JK26, JK28 and JK39. The final concentrations of the peptides were 2.5 µg/ml for JK10, 25 µg/ml for JK49 and 0.25 µg/ml for JK26, JK28 and JK39. Numbers in bold indicate convincing levels of lysis.

<table>
<thead>
<tr>
<th>Peptide (aa in NS3)</th>
<th>Specific 51Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JK10</td>
</tr>
<tr>
<td>#3 (213–227)</td>
<td>0</td>
</tr>
<tr>
<td>#9A (243–257)</td>
<td>0</td>
</tr>
<tr>
<td>#4 (251–265)</td>
<td>47</td>
</tr>
<tr>
<td>#5 (258–272)</td>
<td>21</td>
</tr>
<tr>
<td>#6 (266–280)</td>
<td>0</td>
</tr>
<tr>
<td>#7 (273–287)</td>
<td>0</td>
</tr>
<tr>
<td>#8 (281–295)</td>
<td>ND</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>Dengue-3 antigen</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Experiment not done.
Table 3. Definition of the smallest peptide recognized by CD4+ CTL clones

Effector:target ratios were 5:1 for JK39, 8:1 for JK10 and JK28, and 10:1 for JK26.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>Specific ⁵¹Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JK10</td>
</tr>
<tr>
<td>4</td>
<td>HTGREIVDLCHATF</td>
<td>38</td>
</tr>
<tr>
<td>4c</td>
<td>REIVDLCHATF</td>
<td>–</td>
</tr>
<tr>
<td>4d</td>
<td>EIVDLCHATF</td>
<td>25</td>
</tr>
<tr>
<td>4e</td>
<td>IVDLCHATF</td>
<td>14</td>
</tr>
<tr>
<td>4f</td>
<td>HTGREIVDLCHAT</td>
<td>16</td>
</tr>
<tr>
<td>4g</td>
<td>HTGREIVDLCHA</td>
<td>1</td>
</tr>
<tr>
<td>4h</td>
<td>HTGREIVDLCHM</td>
<td>–</td>
</tr>
<tr>
<td>4k</td>
<td>EIVDLCHAT</td>
<td>32</td>
</tr>
<tr>
<td>4l</td>
<td>EIVDLCHM</td>
<td>1</td>
</tr>
<tr>
<td>4m</td>
<td>IVDLCHAT</td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

* Final concentration of peptides (µg/ml).

#4, while JK49 recognized only peptide #5. JK10 and JK39 recognized peptides #4 and #5 at this peptide concentration; however, a peptide dose-response study showed that JK10 and JK39 recognized peptide #4 to a higher degree than peptide #5 at lower concentrations (data not presented).

Definition of the smallest peptide recognized by CD4 CTL clones

CD4+ CTL clones were examined for recognition of the N- or C-terminal truncations of peptide #4 in order to define the smallest unit of the peptide epitope (Table 3). Peptide 4k, which is comprised of aa 255–264 (EIVDLMCHAT), was the smallest peptide recognized by JK10, JK26, JK28 and JK39. We have previously reported that peptide 4k was also the smallest peptide recognized by JK34 (Kurane et al., 1993). The smallest peptide recognized by JK49 was determined to be peptide D4.NS3.257–266 which comprises aa 257–266 of NS3 (data not presented).

HLA-DPw2-restriction in recognition of the epitope

HLA restriction in the recognition of these epitopes was determined using HLA-defined allogeneic target cells in cytotoxicity assays (Table 4). Allogeneic target cells which share HLA-DPw2 were lysed in the presence of peptide #4 by JK10, JK26, JK28 and JK39, but none of the allogeneic target cells which do not share HLA-DPw2 were lysed. HLA-DPw2-shared, peptide #5-pulsed allogeneic target cells were lysed by JK49. These results and our previous report (Kurane et al., 1993) indicate that all of these six CD4+ CTL clones recognize the epitope in an HLA-DPw2-restricted fashion.

Determination of TCR V gene usage by T cell clones

We determined TCR V gene usage by these CD4+ CTL clones using PCR with 25 Vα-specific primers and 26 Vβ-specific primers. Table 5 shows a summary of the analysis. JK10 and JK34 use Vα11. JK34 and JK28 use Vβ23. It is of interest that the two T cell clones JK10 and JK34 have the same flavivirus specificities (Table 1). TCR Vα usage by JK39 and JK49 could not be determined using 25 Vα-specific primers.

Amino acid sequences of CDR3 regions of TCR α and β chains

It is generally accepted that CDR3 of TCR α and β chains is responsible for recognition of the epitope by T cells (Davis & Bjorkman, 1988). Therefore, we sequenced CDR3 of the TCRs of five CD4+ CTL clones which recognize peptide D4.NS3.255–264 in an HLA-DPw2-restricted fashion. The other dengue virus-specific CD4+ CTL clones, JK1, JK32, JK37, JK38, and JK49, which do not recognize peptide D4.NS3.255–264 were also analysed for comparison.

JK10, JK34 and JK28 have valine (V) following alanine (A) in the CDR3 of the TCR α chain (Fig. 1). This motif was not present in JK32 or JK37, or in JK4 and JK43 which are also flavivirus-cross-reactive but recognize the epitope between aa 146–154 in an HLA-DR15-restricted manner (Kurane et al., 1995). Two TCR β chains were sequenced with JK28. Arginine (R) was present in the VD junctional region of the TCR β chain of JK10, JK34, JK39 and JK28 (Fig. 2). Proline (P) was present in the VD junctional region of the TCR of JK34 and JK28, and glutamine (Q) was present in the same region of the TCR β chain of JK10 and JK39. These amino acids were not present in
Table 4. HLA-DPw2-restricted recognition of peptide 4 by CD4⁺ CTL clones

Effector:target ratios were 6:1 for JK39, 8:1 for JK26 and JK28, and 10:1 for JK10 and JK49. Numbers in bold indicate convincing levels of lysis.

<table>
<thead>
<tr>
<th>Target cell</th>
<th>DR</th>
<th>HLA</th>
<th>Specific ⁵¹Cr release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>w₂</td>
<td>DP</td>
<td>DQ</td>
</tr>
<tr>
<td>Autologous</td>
<td>15 ( = 2), 103</td>
<td>w₂</td>
<td>5 ( = 1), 6 ( = 1)</td>
</tr>
<tr>
<td>9029</td>
<td>4</td>
<td>w₂</td>
<td>8</td>
</tr>
<tr>
<td>9074</td>
<td>9</td>
<td>w₂, w₅</td>
<td>9</td>
</tr>
<tr>
<td>3103</td>
<td>4, 4</td>
<td>w₂</td>
<td>ND</td>
</tr>
<tr>
<td>3106</td>
<td>5</td>
<td>w₂</td>
<td>1</td>
</tr>
<tr>
<td>9038</td>
<td>12</td>
<td>w₂</td>
<td>7</td>
</tr>
<tr>
<td>9049</td>
<td>7</td>
<td>w₁</td>
<td>2</td>
</tr>
<tr>
<td>9087</td>
<td>3</td>
<td>w₃, w₄</td>
<td>2</td>
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<tr>
<td>MS</td>
<td>1, 4</td>
<td>w₄</td>
<td>1, 3</td>
</tr>
<tr>
<td>3107</td>
<td>2</td>
<td>w₄</td>
<td>2</td>
</tr>
<tr>
<td>9077</td>
<td>9, 12</td>
<td>w₅</td>
<td>3</td>
</tr>
</tbody>
</table>

*+, Lysis with peptide g₄ for JK10, JK26, JK28 and JK39, and with peptide D₄.NS3.257–266 for JK49. —, Lysis without peptide. ND, Experiment not done.

Table 5. TCR V gene usage by CD4⁺ CTL clones which recognize peptides #4 or #5

TCR V gene usage was determined by the RT–PCR method using the 25 V α-specific and 26 V β-specific primer sets as described in Materials.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Smallest peptide recognized (aa in NS3)</th>
<th>HLA Restriction</th>
<th>Vα</th>
<th>Vβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK10</td>
<td>255–264</td>
<td>DPw₂</td>
<td>11</td>
<td>13·2</td>
</tr>
<tr>
<td>JK34</td>
<td>255–264</td>
<td>DPw₂</td>
<td>11</td>
<td>23</td>
</tr>
<tr>
<td>JK39</td>
<td>255–264</td>
<td>DPw₂</td>
<td>ND</td>
<td>13·1</td>
</tr>
<tr>
<td>JK28</td>
<td>255–264</td>
<td>DPw₂</td>
<td>2</td>
<td>4 and 23</td>
</tr>
<tr>
<td>JK26</td>
<td>255–264</td>
<td>DPw₂</td>
<td>17</td>
<td>5·2</td>
</tr>
<tr>
<td>JK49</td>
<td>257–266</td>
<td>DPw₂</td>
<td>ND</td>
<td>9</td>
</tr>
</tbody>
</table>

ND, Experiment not done.

the VD junctional region of the TCR of other dengue virus-specific CTL clones.

Discussion

We have shown that five dengue virus-specific CD4⁺ CTL clones which were established form one dengue virus-immune donor recognized peptide D₄.NS3.255–264 on NS3 in an HLA-DPw2-restricted manner. (i) Three T cell clones, JK10, JK34 and JK39, were cross-reactive for dengue virus types 1–4, but were not cross-reactive for WNV or YFV. (ii) One T cell clone, JK28, was cross-reactive for dengue virus types 1–4 and WNV, but was not cross-reactive for YFV. (iii) The other T cell clone, JK26, was cross-reactive for dengue virus types 1–4, WNV and YFV. The amino acid sequences between aa 255 and 264 on NS3 are completely conserved among the four dengue virus serotypes. WNV has one amino acid substitution from dengue viruses, L to V at aa 259 (Castle et al., 1985; Wengler et al., 1985). YFV has three substitutions from dengue viruses, I to V at aa 256, V to I at aa 257 and L to A at aa 259 (Rice et al., 1985). These limited amino acid substitutions appear to contribute to the presence of flavivirus-cross-reactive T cell clones such as JK26 or JK28. The other flavivirus-cross-reactive clone, JK49, recognized the peptide which comprised aa 257–266 in an HLA-DPw2-restricted fashion. Thus, the region on NS3 which comprises aa 255–266
contains multiple epitopes recognized by dengue serotype-cross-reactive and flavivirus-cross-reactive CD4+ T lymphocytes.

Because the five T cell clones, JK10, JK34, JK39, JK26 and JK28, recognize the same smallest epitope in an HLA-DPw2-restricted manner, the differences in flavivirus-specificities of these T cell clones are probably due to differences in recognition of the peptide by TCRs. We sequenced the TCR of these T cell clones in order to understand the interactions between the TCR and the peptide. It is accepted that the V(D)J junctional region of the α and β chains of TCR are responsible for recognition of the peptide. Kalams et al. (1996) reported that HIV-1-specific, HLA-B14-restricted CTL clones which recognized the same epitope used heterogeneous TCRs. We found that amino acid sequences of the CDR3 region of the TCR are different among our five T cell clones. However, there are some conserved amino acids. The α chains of JK10, JK34 and JK28 share valine. The β chains of JK10, JK34, JK39 and JK28 share arginine, and the β chains of JK10 and JK39 share glutamine. Jorgensen et al. (1992) reported results that suggest there is one amino acid in the V(D)J junctional region which is very important for peptide contact. We therefore hypothesize

### Table 1: Sequences of the CDR3 portion of the TCR α chains of CTL clones.

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<thead>
<tr>
<th>V</th>
<th>N</th>
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<tbody>
<tr>
<td>JK10</td>
<td>TACTACTGTCCT</td>
<td>GYGGAGGATCA</td>
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<tr>
<td></td>
<td>YCA</td>
<td>VEDQ</td>
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<tr>
<td>JK34</td>
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<td>GYGGAGGATCA</td>
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<td>JK39</td>
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### Table 2: Sequences of the CDR3 portion of the TCR β chains of CTL clones.

<table>
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<td>JK10</td>
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</table>
that the conserved single amino acids among dengue virus-specific T cell clones are important for recognition of the same peptide. There are, however, other reports which suggest the importance of Vβ and Vβ of the TCR in recognition of the peptides (Boitel et al., 1992; Bellio et al., 1994). JK10 and JK34 use Vα11, JK10 and JK39 use Vβ13, and JK34 and JK28 use Vβ23. The usage of the same Vα or Vβ by some of the T cell clones may also contribute to the recognition of the same peptide or restriction by the same HLA.

These five CD4+ T cell clones are, on the other hand, heterogeneous in flavivirus specificities. Therefore, there should be some differences in recognition of the peptide by the TCR on these T cell clones. The amino acid at the position 259 is different among dengue viruses, WNV and YFV; therefore, the amino acid at position 259 may be a critical residue which contacts the TCR. If this is the case, the TCR of JK10, JK34 and JK39 may be very stringent and discriminate the difference between L and V or A. The TCR of JK26 may be less stringent and does not discriminate these differences. The other possibility is that the critical residue is located at another position and is conserved among dengue viruses, WNV and YFV; however, the binding of these peptides and the exposure of the critical residue are different because of the substitution of amino acids at the other positions (Tussey et al., 1994). If the conserved amino acid in the V(D)j junctional region is the key residue for peptide contact, the latter hypothesis is likely to be correct.

Two TCR β chains were detected with JK28. The amino acid sequences of the two TCRs are both in-frame. Because no monoclonal antibodies to Vβ4 or Vβ23 are available, we cannot determine which β chain is expressed and functional. It has been reported that T cells can express two functionally active TCRs (Hardardotriv et al., 1995). Thus, it is possible that both β chains of CD28 are expressed and functional.

The Flaviviridae includes at least 66 viruses, and there are many regions of the world where more than one flavivirus are prevalent. It is not clearly known whether immunity to one flavivirus provides protection against other flaviviruses, or contributes to immunopathology as is observed in dengue haemorrhagic fever. The presence of flavivirus-cross-reactive CD4+ T lymphocytes such as JK26, JK28 and JK49 suggest that memory T lymphocytes induced by primary flavivirus infections can be activated during secondary flavivirus infections, and may modulate the outcome of the secondary infections. These flavivirus-cross-reactive T cell clones have different flavivirus specificities but still recognize the same peptide. It was reported that lymphocytic choriomeningitis virus-specific, murine CTL clones which had different strain specificities recognized the same peptide (Joly et al., 1989). Thus, this phenomenon is not unique to dengue virus-specific T cell responses, but also seems to be observed in other virus infections. These CTL clones provide an interesting model for detailed understanding of the interactions between the TCR and the peptide. In addition, it suggests that development of T-cell-based vaccines which are effective against multiple flaviviruses may be possible.

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References


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