Partial Agonist Effect Influences the CTL Response to a Heterologous Dengue Virus Serotype

Jaroslav Zivny, Matthew DeFronzo, William Jarry, Julie Jameson, John Cruz, Francis A. Ennis and Alan L. Rothman

*J Immunol* 1999; 163:2754-2760; 
http://www.jimmunol.org/content/163/5/2754

References

This article cites 45 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/163/5/2754.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Partial Agonist Effect Influences the CTL Response to a Heterologous Dengue Virus Serotype

Jaroslav Zivny, Matthew DeFronzo, William Jarry, Julie Jameson, John Cruz, Francis A. Ennis, and Alan L. Rothman

Activation of dengue serotype-cross-reactive memory CTL during secondary dengue virus (DV) infection is thought to be important in the pathogenesis of dengue hemorrhagic fever. To model this effect, we studied the CTL responses to DV types 2 (D2V) and 3 (D3V) in PBMC from an individual previously infected with D3V. DV-specific CD8+ CTL from this donor recognized two HLA-B62-restricted epitopes on the NS3 protein, aa 71–79 (SVKKDLISY) and 235–243 (AMKGLPIRY). Both D3V-specific and D2V/D3V-cross-reactive CTL clones were detected for each epitope; all D2V-reactive CTL clones could lyse D2V-infected autologous cells. CTL responses to both epitopes were detected in bulk cultures stimulated with D3V, but PBMC stimulated with D2V recognized only the 235–243 epitope. IFN-γ enzyme-linked immunospot assay showed that the D2V (71–79) peptide (DVKKDLISY) did not efficiently activate T cells. Analysis of a CTL clone suggests that the D2V (71–79) peptide acts as a partial agonist, able to sensitize target cells for lysis and inducing only minimal proliferation at high concentrations. These results suggest that variant peptide sequences present in the heterologous DV serotype can influence the CTL response in vivo during secondary DV infection.


Infection by any of the four serotypes of dengue virus (DV), D1V, D2V, D3V, and D4V, can cause relatively benign illness but can also result in a life-threatening disease characterized by increased vascular permeability known as dengue hemorrhagic fever (DHF) (1, 2). The host- and virus-specified factors that cause DHF are not fully understood. However, studies from Thailand, Cuba, and Myanmar have demonstrated that the frequency of DHF is 15–80 times higher in secondary DV infections than in primary DV infections (3–6). There is a theoretical basis for the contribution of serotype-cross-reactive Abs or serotype-cross-reactive T lymphocytes (or both), induced by primary DV infection, to this phenomenon (7–10).

A role for T lymphocyte activation in the pathogenesis of DHF is supported by the finding that the levels of IFN-γ, soluble CD8, and soluble IL-2 receptors in the blood are markedly elevated in children with DHF even before the onset of plasma leakage (11, 12). Studies of volunteers immunized with single-serotype, live, experimental DV vaccines have detected a high frequency of serotype-cross-reactive CD8+ T cells in bulk culture assays, limiting dilution assays, and at the clonal level (13, 14). CD8+ CTL have been studied in a more limited way in bulk culture and at the clonal level (15–18). Serotype-cross-reactive CD8+ T cells were detected; however, in previous studies, DV-specific CTL were generated by in vitro stimulation with the same serotype of DV that the donor had been immunized with.

Activation of CTL by heterologous virus stimulation may better reflect the in vivo situation, where secondary infection always involves a different serotype than the primary infection. Amino acid sequence homology between the different DV serotypes is ~65–75% and varies among the different regions of the polyprotein (19). As a result, there is often not complete sequence homology at the epitopes recognized by serotype-cross-reactive T cells (16, 18, 20–25). Other investigators have shown that the introduction of amino acid substitutions in antigenic peptides can induce a variety of responses in peptide-specific T cells, ranging from full activation through partial activation and indifference to antagonism (26–28). Partial agonist peptides may induce the full complement of functional responses at a lower level or may induce only some of the functional responses of the peptide-specific T cells.

Interactions of T cells with such altered peptide ligands have been proposed to play a role in thymocyte development and may have some use in the treatment of autoimmune diseases (26–28). There is limited data on the potential role of altered peptide ligands in host-pathogen interactions. Viral mutations that generate T cell antagonist or partial agonist peptides have been described in humans chronically infected with HIV, hepatitis C virus, and hepatitis B virus and have been proposed to facilitate viral persistence (29–31). However, no examples of natural altered peptide ligands in acute viral infections of humans have been described previously.

We show that stimulation of PBMC of a D3V-immune donor by a heterologous dengue serotype can activate memory CTL in vitro. We also extend the limited observations on CD8+ CTL clones in DV-immune donors by mapping two new epitopes on the NS3 protein. At the bulk culture level, stimulation with homologous D3V led to activation of CTL that recognized both CTL epitopes. However, stimulation with D2V led to activation of CTL that recognized only one of the CTL epitopes. Experiments with peptide-stimulated bulk cultures and CTL clones suggest that the D2V sequence at the second CTL epitope acts as a partial agonist.

Received for publication January 20, 1999. Accepted for publication June 11, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by Grant R01 AI30624 from the National Institute of Allergy and Infectious Diseases. The opinions expressed are those of the authors and do not necessarily reflect the opinions of the National Institute of Allergy and Infectious Diseases.

Address correspondence and reprint requests to Dr. Alan L. Rothman, Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School, Worcester, MA 01655.

Abbreviations used in this paper: DV, dengue virus; DHF, dengue hemorrhagic fever; LCL, lymphoblastoid cell line; huABS, human AB serum; ELISPOT, enzyme-linked immunospot; SI, stimulation index.
Materials and Methods

Viruses

D2V (New Guinea C strain) and D3V (CH53489 strain) were propagated as previously described and frozen at −70°C until use (32). Recombinant vaccinia viruses expressing D2V and D3V NS3 proteins and their truncations were provided by Dr. M. Briton (Georgia State University, Atlanta, GA), and the vaccinia virus expressing D4V NS3 protein was provided by Dr. C. J. Lai (National Institutes of Health, Bethesda, MD) (23).

Human cells

A peripheral blood specimen was obtained from a healthy adult who had been immunized with yellow fever vaccine 2 years earlier and with D3V (CH53489) vaccine 1 year earlier (33). PBMC were cryopreserved until use.

Bulk culture of PBMC

PBMC were suspended at 3.3 × 10^6 cells/ml in AIM-V medium (Life Technologies, Gaithersburg, MD) containing 10% heat-inactivated AB serum (huABS; Advanced Biotechnologies, Columbia, MD). Then, 5 × 10^5 cells in 1.5 ml were added to 0.5 ml of D2V or D3V in 24-well cluster plates (Costar, Cambridge, MA) as previously described (15). Cytolytic activity was assayed using these cells after 8–9 days of culture at 37°C. In bulk cultures stimulated with dengue peptides, 6 × 10^4/ml of D2V-infected Raji cells were maintained in cultures for 2–6 wk before use as CTL targets. Between 50 and 70% of D2V-infected LCL contained DV Ag as detected by immunofluorescence using hyperimmune anti-D2V mouse ascites fluid.

Cytotoxicity assays

Cytotoxicity assays were performed in 96-well round-bottom plates as previously described (14, 20). Effector cells in 0.1 ml RPMI 1640 containing 10% FCS were added to 2 × 10^3 51 Cr-labeled target cells in 0.1 ml at E:T ratios 20–100:1 for bulk cultures and 10:1 for CTL clones. In cytotoxicity assays with CTL clones and synthetic peptides, 0.05 ml of peptide was added to 2 × 10^5 target cells in 0.1 ml, incubated at 37°C for 30 min, and 0.05 ml of effector cells were then added. In cytotoxicity assays with bulk cultures and synthetic peptides, target cells were first incubated with 1–10 μg/ml of synthetic peptide for 30 min, then labeled with 51 Cr for 18 h at 37°C. Supernatant fluids were collected with the supernatant collection system (Skatron Instruments, Sterling, VA), and the 51 Cr content was measured in a gamma counter (Packard Instruments, Sterling, CA). Maximum 51 Cr release was determined from wells containing target cells and the minimal peptide concentration (0.1 μM); background 51 Cr release was determined from wells containing target cells and medium only. Percent specific lysis was calculated as (experimental 51 Cr release − minimum 51 Cr release) ÷ (maximum 51 Cr release − minimum 51 Cr release) × 100. Assays were performed in triplicate wells. The SEM of samples did not exceed 10%. Minimum 51 Cr release was generally <25% of the maximum 51 Cr release.

Proliferation assay

Proliferation assay with CTL clones was done by modification of a previously described method (36). On day 14 after restimulation, cells were washed once and cultured for 5 days in AIM-V containing 10% FBS. Then, 2 × 10^5 clone cells per well were seeded in 96-well round-bottom plates with 0 × 10^5 or 10 × 10^5 PBMC (responders) plus 1 × 10^6 α-irradiated autologous PBMC (stimulators) preincubated with D3V (multiplicity of infection − 1) in a final volume of 200 μl/well AIM-V containing 10% huABS. Twenty replicate wells were prepared for each condition. After incubation for 7 days, 50 μl was removed from each well and transferred to each of three replicate plates. Autologous B-LCL were incubated with the D3 (71–79) or D2 (71–79) peptides (10 μg/ml) or medium alone, labeled with 51 Cr, washed, and added at 1500 cells/well with 1.5 × 10^6 unlabeled K562 cells/well. After centrifugation, plates were incubated for 4 h and harvested as above. Individual wells were scored as positive for peptide-specific lysis when the lysis of peptide-pulsed target cells was >10% and exceeded lysis of nonpeptide-pulsed target cells by at least 20%. Wells showing >20% lysis of control target cells were excluded from analysis.

Results

Stimulation of memory dengue NS3-specific CTLs by heterologous serotypes of DV

We previously reported that CD4+ T lymphocytes present in PBMC of a D3V-immunized volunteer proliferated in response to D3V Ags as well as Ags of the other dengue serotypes (14). We also reported that bulk culture CTL generated from this donor’s PBMC by stimulation with D3V recognized one or more epitopes on the dengue NS3 protein in a serotype-cross-reactive manner (17). Because a secondary DV infection in this donor would be expected to involve a serotype other than D3V, we compared the cytolytic activity of PBMC stimulated with D2V or D3V against autologous B-LCL infected with recombinant vaccinia viruses expressing the D2V, D3V, or D4V NS3 proteins (Table 1). CTL generated by stimulation with D3V lysed target cells expressing the D2V and D3V NS3 proteins, and, to a lesser extent, the D4V...
NS3 protein. CTL generated by stimulation with D2V lysed target cells expressing D2V, D3V, and D4V NS3 proteins. These data indicate that DV serotype-cross-reactive CTL from PBMC from a D3V-immune donor are activated by stimulation with a heterologous dengue serotype.

Characterization of DV-specific CD8$^+$ CTL clones

After limiting dilution of PBMC stimulated in vitro with D2V or D3V, we isolated eight CD3$^+$ CD4$^-$ CD8$^+$ CTL clones that demonstrated DV NS3-specific cytolytic activity. Seven of the eight CTL clones (JK1, JK19, JK30, JK38, JK41, JK62, and JK65) were obtained from the bulk culture originally stimulated with D3V. Clone JK119 was obtained from the bulk culture originally stimulated with D2V. Three distinct patterns of DV serotype recognition were observed using autologous B-LCL target cells infected with recombinant vaccinia viruses expressing the D2V, D3V, or D4V NS3 protein (Table II). Clones JK1, JK30, and JK65 were specific for D3V. Clones JK41 and JK62 were cross-reactive for D2V and D3V, but did not recognize D4V. Clones JK19, JK38, and JK119 were cross-reactive for D2V, D3V, and D4V.

We localized the epitopes recognized by these CTL clones using target cells infected with recombinant vaccinia viruses expressing truncations of the NS3 protein. Clones JK1, JK19, JK38, and JK119 recognized target cells expressing aa 1–247 but not 1–216 of NS3, demonstrating that the epitope is located between aa 216 and 247 (data not shown). Clones JK30, JK41, JK62, and JK65 recognized a recombinant vaccinia virus expressing aa 1–83 of NS3 (data not shown). We then tested for recognition by the CTL clones of synthetic peptides spanning these regions, initially using overlapping peptides of 15–20 aa and then truncations of peptides showing recognition (data not shown). The peptide D3-NS3 (235–243; AMKGLPIRY) was the shortest peptide recognized by clones JK1, JK19, JK38, and JK119, and the peptide D3-NS3 (71–79; SVKKDLISY) was the shortest one recognized by clones JK30, JK41, JK62, and JK65 (Fig. 1).

The HLA restriction of these CTL clones was determined using a panel of partially HLA-matched autologous B-LCL. All eight CTL clones lysed recombinant vaccinia virus-infected or peptide-pulsed autologous target cells that shared HLA-B62 (Table III). Wild-type vaccinia virus-infected or uninfected, unpulsed autologous B-LCL were not lysed by the clones (data not shown). These results demonstrate that all CD8$^+$ CTL clones are HLA-B62-restricted.

To confirm that these CD8$^+$ CTL clones lyse DV-infected target cells, we examined the ability of the CTL clones to lyse autologous LCL persistently infected with D2V. The serotype-cross-reactive CTL clones JK19, JK38, JK41, JK62, and JK119 lysed D2V-infected autologous LCL, whereas, as expected, the D3V-specific CTL clones JK1, JK30, and JK65 did not lyse these target cells (Table IV).

Recognition of NS3 epitopes by T cells in short-term bulk cultures

To determine whether the CTL clones we isolated were representative of the bulk culture DV-specific CTL response in this donor, we tested for recognition of these peptides by DV-stimulated CTL in bulk culture. CTL generated after stimulation with D3V lysed target cells pulsed with any of the four peptides D2-NS3 (71–79), D3-NS3 (71–79), D2-NS3 (234–242), or D3-NS3 (235–243) (Table V). However, CTL generated after stimulation with D2V recognized only target cells pulsed with the peptides D2-NS3 (234–242) and D3-NS3 (235–243) and did not lyse target cells pulsed with D2-NS3 (71–79) or D3-NS3 (71–79) (Table V).

To confirm these observations with virus-stimulated bulk cultures, we examined the CTL activity of bulk cultures stimulated with either the D2 or D3 variants of both of these peptide epitopes. Each of the peptides, with the exception of the peptide D2-NS3 (71–79), stimulated CTL in bulk culture that lysed autologous target cells infected with recombinant vaccinia viruses expressing the corresponding epitope of both D2V and D3V NS3 proteins (Table VI).
These results indicate that CTL capable of recognizing both of these epitopes are present in virus- or peptide-stimulated bulk cultures. However, whereas CTL specific for the peptide D2-NS3 (234–242) are activated by stimulation with heterologous virus (D2V) or the corresponding peptide, CTL directed against the peptide D2-NS3 (71–79) are not activated by such stimulation.

To further test the hypothesis that the D2-NS3 (71–79) peptide was not able to activate the memory CD8\(^+\) T cells in this donor, we used an IFN-\(\gamma\) ELISPOT method to measure the frequency of peptide-responsive cells in the PBMC of this donor. The frequencies of IFN-\(\gamma\)-producing cells after stimulation with the peptides D3-NS3 (71–79), D2-NS3 (234–242), and D3-NS3 (235–243) were 1 in 40,000, 1 in 150,000, and 1 in 130,000 cells, respectively. In contrast, we did not detect IFN-\(\gamma\)-producing cells (<1 in 500,000) after stimulation with the peptide D2-NS3 (71–79). For comparison, we measured the frequency of CTL precursors to the D3-NS3 (71–79) and D2-NS3 (71–79) peptides after stimulation with D3V using limiting dilution analysis. As has been reported in other systems (39, 40), the frequency of D3-NS3 (71–79)-specific CTL measured in limiting dilution analysis (1 in 171,000) was lower than that obtained from IFN-\(\gamma\)-ELISpot assay. Three of 20 wells demonstrating recognition of the D3-NS3 (71–79) peptide also recognized the D2-NS3 (71–79) peptide. These results confirm that the D2-NS3 (71–79) peptide was unable to activate memory T cells in this donor’s PBMC for IFN-\(\gamma\) production, even though memory CTL cross-reactive with this peptide could be activated by D3V stimulation.

Partial dissociation of cytotoxicity and proliferation in a CTL clone specific for the NS3 (71–79) epitope

The bulk culture results presented above suggested that peptide D2-NS3 (71–79) may act as partial agonist, inducing a cytotoxic response but unable to induce a proliferative response or IFN-\(\gamma\) production. To test this hypothesis, we compared the ability of this peptide to induce cytolysis and proliferation by the serotype cross-reactive clone JK41 (Table VII). Clone JK41 lysed target cells pulsed with peptide D2-NS3 (71–79) at concentrations \(\leq 25 \mu\text{g/ml}\). Comparable levels of lysis were induced with 100-fold less of the D3-NS3 (71–79) peptide. However, even at these high concentrations, the D2-NS3 (71–79) peptide induced only minimal proliferation by clone JK41, with a maximum SI of 4 at a peptide concentration of 25 \(\mu\text{g/ml}\). In contrast, the maximum SI of this clone stimulated by the D3-NS3 (71–79) peptide was 28 at a concentration of 25 \(\mu\text{g/ml}\), and the SI was 8 at a peptide concentration 500,000 after stimulation with the peptide D2-NS3 (71–79). For comparison, we measured the frequency of CTL precursors to the D3-NS3 (71–79) and D2-NS3 (71–79) peptides after stimulation with D3V using limiting dilution analysis. As has been reported in other systems (39, 40), the frequency of D3-NS3 (71–79)-specific CTL measured in limiting dilution analysis (1 in 171,000) was lower than that obtained from IFN-\(\gamma\)-ELISpot assay. Three of 20 wells demonstrating recognition of the D3-NS3 (71–79) peptide also recognized the D2-NS3 (71–79) peptide. These results confirm that the D2-NS3 (71–79) peptide was unable to activate memory T cells in this donor’s PBMC for IFN-\(\gamma\) production, even though memory CTL cross-reactive with this peptide could be activated by D3V stimulation.

**FIGURE 1.** Recognition of peptides derived from the D3V NS3 protein by CD8\(^+\) CTL clones. Autologous B-LCL target cells (2 \(\times\) 10\(^3\)/well) were incubated with effector cells in the presence of the relevant peptide at the indicated concentrations in a 4-h cytotoxicity assay. The E:T ratio was 10:1 for all clones. **A.** Recognition of peptide D3-NS3 (235–243; AMIKGLPIRY) by CTL clones JK1, JK19, JK38, and JK119. **B.** Recognition of peptide D3-NS3 (71–79; SVKKDLISY) by clones JK30, JK41, JK62, and JK65.
of 0.25 μg/ml. As expected, the serotype-specific clone JK65 did not show any cytotoxic or proliferative response to the D2 peptide. We interpret these results to show a partial dissociation of the cytotoxicity and proliferative responses of clone JK41 to the D2 peptide.

Discussion

The findings in the present study expand upon existing knowledge of the CD8$^+$ CTL response to DV. In so doing, we found that stimulation by a heterologous DV serotype induced only partial activation in a subpopulation of serotype-cross-reactive T cells. Although partial agonist effects of altered peptide ligands have been previously documented (26–28), to our knowledge this is the first association of this phenomenon with an acute viral infection.

We isolated eight CD8$^+$ CTL clones specific for the DV NS3 protein from a donor who was immunized with D3V. We identified two HLA-B62-restricted CTL epitopes on the NS3 protein, residues 71–79 and 235–243, that had not previously been identified. We isolated both DV serotype-specific and serotype-cross-reactive CTL specific for each of these epitopes. Although this donor also has the HLA-B7 allele, we did not isolate any CD8$^+$ CTL clones that recognized the HLA-B7-restricted NS3 (222–230) epitope, which was described previously (18).

Our laboratory has previously identified six epitopes on the NS3 protein that are recognized by CD4$^+$ T cells from donors infected with D2V or D3V. One CD4$^+$ epitope, NS3 (241–249), overlaps the C terminus of one of the CD8$^+$ epitopes, and another CD4$^+$ epitope, NS3 (224–234), is adjacent to the N terminus of the same CD8$^+$ epitope. Immunodominance of the flavivirus NS3 protein has been suggested by these and other studies in humans and mice (16–18, 41, 42). Lobigs et al. proposed that the localization of NS3 synthesis to the cytoplasmic surface of the endoplasmic reticulum may facilitate peptide entry into the MHC class I presentation pathway (43). However, the predominance of recognition of the flavivirus NS3 protein by both CD4$^+$ and CD8$^+$ T cells suggests that peptide translocation into the endoplasmic reticulum does not fully explain its immunogenicity.

To assess the potential relevance of the observations made using CTL clones, we tested for recognition of these two CTL epitopes by DV-stimulated bulk cultures of PBMC, as previously reported for CD4$^+$ CTL epitopes (22). In keeping with the data obtained using the CTL clones, we found that PBMC stimulated with the DV serotype with which this donor was infected (D3V) recognized both CTL epitopes in a serotype-cross-reactive fashion. However, while stimulation of this donor’s PBMC with a heterologous DV serotype (D2V) did activate serotype-cross-reactive memory CTL specific for the NS3 (235–243) epitope, there was no detectable response to either the D2V or D3V sequences of the NS3 (71–79) epitope in this bulk culture.

Our studies suggest that the memory CD8$^+$ CTL directed at the NS3 (71–79) epitope that were induced by D3V immunization in this donor are only partially activated by the D2-NS3 (71–79) sequence, although there is only a single amino acid difference (S → D at position 1) between the two peptides. As a result, the sero-
type-cross-reactive CTL specific for this epitope do not proliferate enough in bulk culture to be detectable in cytotoxicity assays. The results of the IFN-γ ELISPOT assay also suggest that stimulation by the D2-NS3 (71–79) peptide is inadequate to induce IFN-γ production by these clones. Experiments with CTL clone JK41 showed that higher concentrations of the D2-NS3 (71–79) peptide were required to sensitize target cells for lysis by this clone and that even high concentrations of this peptide did not induce strong proliferation of this clone.

---

Table V. Recognition of DV NS3 peptides by D2V- and D3V-stimulated bulk cultures

<table>
<thead>
<tr>
<th>In Vitro Stimulation</th>
<th>% Specific Lysis of Target Cells Pulsed with Indicated Peptide$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2V</td>
<td>3</td>
</tr>
<tr>
<td>D3V</td>
<td>16</td>
</tr>
</tbody>
</table>

$^a$ PBMC (4 × 10⁶) were incubated for 9 days in the presence of D2V or D3V.

Table VI. Recognition of DV proteins by peptide-stimulated bulk cultures

<table>
<thead>
<tr>
<th>In Vitro Stimulation</th>
<th>E/T Ratio</th>
<th>% Specific Lysis of Target Cells Infected with Indicated Vaccinia Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2-NS3 (71–79)</td>
<td>80:1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>1</td>
</tr>
<tr>
<td>D3-NS3 (71–79)</td>
<td>80:1</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>21</td>
</tr>
<tr>
<td>D2-NS3 (234–242)</td>
<td>80:1</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>5</td>
</tr>
<tr>
<td>D3-NS3 (235–243)</td>
<td>80:1</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>20:1</td>
<td>13</td>
</tr>
</tbody>
</table>

$^a$ A total of 4 × 10⁶ γ-irradiated autologous PBMC were pulsed with 10 μg/ml of peptide D2-NS3 (71–79), D3-NS3 (71–79), D2-NS3 (234–242), or D3-NS3 (235–243). The cells were then washed and incubated with 4 × 10⁶ autologous PBMC for 9 days. Recombinant IL-2 was added at 25 U/ml on day 3.

$^b$ A total of 1 × 10⁶ autologous targets were infected with recombinant vaccinia virus that contains genes coding for the full-length NS3 protein of D2V and D3V or truncations coding for aa 1–114 and aa 1–133 of NS3, respectively. Infected targets were incubated with 8 × 10³ (E/T 80) or 2 × 10⁶ (E/T 20) effector cells and 2 × 10⁵ K562 cells for 4 h.
Unfortunately, we cannot accurately determine the frequency of this CTL population in PBMC. Because these CTL do not proliferate well to D2V stimulation, it is impossible to use traditional precursor frequency assays based on D2V stimulation. IFN-γ ELISPOT assays, which have been shown to provide a much more accurate estimate of the frequency of peptide-specific T cells (39, 40), could not be used for the reasons stated above. However, by multiplying the frequency of D3-NS3 (71–79) peptide-specific T cells determined by ELISPOT assay (1/40,000) and the fraction of wells in the limiting dilution analysis showing cross-reactive recognition of the D2-NS3 (71–79) peptide (15%), we estimate this frequency to be 1 in 267,000, which should have been detectable by ELISPOT and bulk culture CTL assays.

It is unlikely that D2V-infected APC fail to present the D2-NS3 (71–79) peptide, because the CTL clones that recognize this peptide can lyse autologous D2V-infected cells. Furthermore, stimulation with the D2-NS3 (71–79) peptide was also ineffective at activating this CTL population, while stimulation with the D3-NS3 (71–79) peptide could do so.

Activation of memory T lymphocytes during secondary DV infections is thought to play a role in the pathogenesis of DHF (10). On the basis of the current results, we hypothesize that partial agonist effects of homologous but nonidentical sequences of the second DV serotype may participate in this phenomenon. Depending upon the range of effector responses activated, partial activation of some serotype-cross-reactive memory T cells could enhance the proinflammatory elements of the T cell response that cause DHF, while at the same time providing a suboptimal antiviral effect. In most studies, the intensity of the signal required to induce different T cell responses has followed the pattern of cytotoxicity < IFN-γ secretion < proliferation (44–46). This is consistent with our data showing that the D2-NS3 (71–79) peptide did not induce IFN-γ production in ELISPOT assays. However, we did not measure the production of other cytokines after stimulation with this peptide.

Rogers et al. demonstrated that the early phase of T cell activation, as measured by induction of CD69 expression, proceeded normally in murine CD4+ T cells stimulated with a partial agonist peptide, and that failure to induce high-level IL-2 production was responsible for the poor proliferative response (47). A similar effect may explain the finding that CD69 was expressed on average by 23% of circulating CD8+ T cells before the onset of plasma leakage in children who developed DHF, a significantly higher percentage than in children with dengue fever (51). We hypothesize that some of the cells in this large population undergoing early stages of activation are responding to a weak stimulus. The cells may produce some proinflammatory cytokines, such as macrophage inflammatory protein-1β, IFN-γ, and TNF-β (48, 49), which contribute to plasma leakage.

 Whereas the serotype-cross-reactive memory T cell response after primary DV infection appears to be directed against a wide variety of epitopes (50), we have found in a small number of individuals who were studied after a secondary DV infection that the DV-specific memory T cell response was directed at very few epitopes (18). This finding may indicate that only a small subset of memory T cells are optimally stimulated by the heterologous DV serotype.

Sequential infection of humans with closely related viruses is not common in nature, but other examples include influenza viruses and rhinoviruses. Our findings suggest that the T lymphocyte responses in these viral infections might also be modified by partial sequence homology and an altered peptide ligand effect.

**Acknowledgments**

We thank Jurand Janus and Anita Leporati for technical assistance.

**References**

Partial agonist peptides in heterologous dengue serotypes


