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T CELL RECEPTOR Vβ GENE USAGE IN THAI CHILDREN WITH DENGUE VIRUS INFECTION

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Abstract. T lymphocyte activation during dengue is thought to contribute to the pathogenesis of dengue hemorrhagic fever (DHF). We examined the T cell receptor Vβ gene usage by a reverse transcriptase-polymerase chain reaction assay during infection and after recovery in 13 children with DHF and 13 children with dengue fever (DF). There was no deletion of specific Vβ gene families. We detected significant expansions in usage of single Vβ families in six subjects with DHF and three subjects with DF over the course of infection, but these did not show an association with clinical diagnosis, viral serotype, or HLA alleles. Differences in Vβ gene usage between subjects with DHF and subjects with DF were of borderline significance. These data suggest that the differences in T cell activation in DHF and DF are quantitative rather than qualitative and that T cells are activated by conventional antigen(s) and not a viral superantigen.

INTRODUCTION

Infection with dengue virus (DV), a mosquito-borne flavivirus, is a major health problem for many tropical and subtropical areas of the world, where as many as 100 million DV infections occur yearly.1 This virus exists as four distinct serotypes;2 infection with one DV serotype induces long-lived immunity against reinfection with that serotype. However, epidemiologic and laboratory studies indicate that re-infection with a heterologous DV serotype is more likely to result in the potentially life-threatening form of disease termed dengue hemorrhagic fever (DHF), characterized by increased capillary permeability.1,3–5 It has been proposed that DHF reflects an immunopathologic response to DV infection.6 Studies have demonstrated that levels of circulating soluble CD8 and soluble interleukin-2 receptors (sIL-2R), markers of immune activation, are higher in children with DHF than in those with dengue fever (DF).7,8 Additionally, a high level of CD69 expression has been observed on circulating peripheral blood mononuclear cells (PBMCs) of children with DHF.9

The biological basis for the increased T cell activation in DHF has not been determined. Expansion of T cell subsets bearing T cell receptors (TCRs) using particular Vβ gene families has been associated with severe disease in other infectious or autoimmune disorders. Specific examples include toxic shock syndrome, leprosy, infection with human immunodeficiency virus (HIV), Crohn’s disease, and reactive arthritis.10–14 In vitro analysis of TCR usage by a panel of DV-specific CD4+ cytotoxic T lymphocyte clones generated from one individual after a primary dengue-4 virus infection demonstrated that eight of 19 clones expressed Vβ17.15 A similar expansion of Vβ 17-expressing cells was observed following bulk culture stimulation of this donor’s PBMCs with D4 antigen but not following stimulation with an anti-CD3 antibody. These data provided preliminary evidence that DV-infection might result in expansion of T cells bearing a particular Vβ chain.

We therefore sought to determine whether an expansion of T lymphocytes expressing a particular Vβ gene occurs in vivo upon DV infection and whether preferential Vβ region usage correlates with disease severity. We analyzed the Vβ gene usage in PBMCs obtained during and after the acute infection from 13 subjects with DHF and 13 with DF using a semi-quantitative polymerase chain reaction (PCR) method.16 We confirmed that this method yields highly reproducible results. We detected expansions of T cells expressing particular Vβ genes during acute DV infection in some subjects. However, we did not find consistent expansions of specific Vβ gene families among the group of subjects as a whole or significant differences in Vβ usage between patients with DHF and those with DF.

MATERIALS AND METHODS

Clinical study design and sample collection. Thai children between the ages of six months and 14 years were enrolled in the Dengue Hemorrhagic Fever Project, as previously reported.17 Written informed consent was obtained from the subjects’ parents or guardians. The study protocol was approved by Institutional Review Boards established by the Ministry of Public Health, Thailand, the Surgeon General’s Office of the Department of the Army, and the University of Massachusetts. We selected at random 13 children with DHF and 13 children with DF from among the study population enrolled between April 1994 and November 1995. Dengue virus infections were confirmed by serologic testing and virus isolation as described.18 Clinical diagnoses of DHF and DF were assigned according to World Health Organization criteria.19,20

Blood samples were obtained daily during illness and at a follow-up visit six months after the acute DV infection.21 The PBMCs were isolated by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO). They were washed and counted, and 2 × 10^6 cells were then plated into an Eppendorf tube (Brinkman Instruments, Westbury, NY) and washed twice with phosphate-buffered saline. Finally, the cells were pelleted and 1.0 ml of solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 2%...
[w/v] sarcosyl, and 0.1 M 2-mercaptoethanol) was added. Samples were then stored at −70°C, shipped to the University of Massachusetts Medical School on dry ice, and kept at −70°C until analysis. We selected for this study blood samples obtained on study day 2 (one day after enrollment), fever day +1 (one day after defervescence), and study day 180 (follow-up visit). All samples were analyzed under code.

Isolation of RNA and first-strand cDNA synthesis. Total cellular RNA was isolated by the acid guanidium-phenol-chloroform method. During this procedure, precipitation with isopropanol was performed with glycogen as a carrier. The RNA was treated with DNaseI for 2–3 hr to degrade the DNA, with RNaGuard (Pharmacia Biotech, Inc., Piscataway, NJ) to prevent RNA degradation. First-strand cDNA was synthesized from this RNA using random hexamer primers (Pharmacia Biotech, Inc.) and Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Gaithersburg, MD) for 4–16 hr, and the reaction was terminated by heating samples at 100°C for 10 min.

Analysis of TCR Vβ gene usage by PCR. Analysis of TCR Vβ gene usage was performed as previously described. Twenty-six TCR β-chain variable region-specific 5′ oligonucleotide primers and one TCR β-chain constant region-specific 3′ oligonucleotide primer were synthesized at the DNA Synthesis Facility at the University of Massachusetts Medical School on the basis of previous reports. β-actin-specific oligonucleotide 5′ and 3′ primers were also prepared. The 3′ primers were end-labeled with 32P using T4 polynucleotide kinase (Promega Corp., Madison, WI) and purified using Chroma Spin-10 columns (Clontech Laboratories, Inc., Palo Alto, CA). A volume of labeled 3′ primer equivalent to 104 counts/min (cpm) was used in each reaction. We used 26 cycles of a PCR at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. In the first cycle, a longer denaturing step (1.5 min) was used. Following the PCR, total reaction mixtures were subjected to electrophoresis on a 5% polyacrylamide gel. The radioactivity of each amplified fragment was examined with a Betascope (Betagen, Mountain View, CA) for 100 min. The percentage of each TCR Vβ subfamily expression was calculated by the following formula: expression of TCR Vβn = 100 × (relative expression of TCR Vβn/sum of the relative expression of all TCR Vβ subfamilies), where relative expression of TCR Vβn = cpm of TCR Vβn product/cpm of β-actin product amplified in the same reaction tube.

If no β-actin product was detected in one or more of the PCRs, the relative expression of that TCR Vβ was determined from a partial repeat analysis of the sample as follows. Reactions that failed to yield a β-actin product were retested with at least six reactions that yielded both β-actin and TCR Vβ products, including some with high and low relative expression. Missing values were then interpolated from the linear regression equation. The median correlation coefficient (r) of repeated analyses was 0.97, with a range of 0.85–1.00.

HLA typing. HLA Class I serotyping was performed on peripheral blood lymphocytes within 24 hr of collection, using a standard two-stage microlymphocytotoxicity test for HLA-A, B, and C, as previously described. HLA class II molecular typing of the DRB1, DQA1, DQB1, and DPB1 gene loci was performed on genomic DNA, using the 11th and 12th International Histocompatibility Workshop primers, sequence-specific oligonucleotide probes and protocols as previously described.

Statistical analysis. Pearson correlation was used to compare the expression of all TCR Vβ gene families in serial analyses of the same PBMC samples. For comparison of TCR Vβ expression in different PBMC samples, percentage values were first transformed using the logit function, f(x) = log(x/(1 − x)), using 2% as a minimum value for expression of each TCR Vβ gene family (to avoid skewing of results because of small absolute changes in expression of low abundance TCR Vβ transcripts). Changes in expression of specific TCR Vβ gene families between two time points for an individual subject were considered significant if the differences in transformed values fell outside the mean ± 3 SD of differences for all TCR Vβ gene families between those two time points. We compared the expression of each TCR Vβ gene family between different time points for the study population as a whole using a paired t-test. We compared the expression of each TCR Vβ gene family at each time point and changes in expression of each TCR Vβ gene family between time points in subjects with DHF with the corresponding values in subjects with DF using a t-test. We set P < 0.01 as the criterion for statistical significance, to adjust in part for the number of comparisons.

RESULTS

Characteristics of the study population. Table 1 shows the clinical, serologic, and virologic information on the study subjects. Five of 13 DF patients had primary DV infections, while all of the DHF patients were experiencing secondary infections. All four serotypes of DV were represented in both the DF and DHF groups. Among the subjects studied with DHF, there were six with DHF grade 1, six with DHF grade 2, and one with DHF grade 3. Data on HLA class I alleles were available for 23 subjects and data on HLA class II alleles were available for 24 subjects (Table 1). The mean age of the subjects studied was 8.5 years (95% confidence interval [CI] = 7.3–9.7), and was not significantly different between those children with DHF (mean = 7.9 years) and those with DF (mean = 9.1 years).

Reproducibility of the PCR assay. We first analyzed the reproducibility of the PCR assay for measurement of TCR Vβ gene usage by repeated analysis of cDNA prepared from seven blood samples. The mean correlation coefficient for the comparison of the results from repeated PCR amplification of the same specimens was 0.97 (95% CI = 0.96–0.99).

Changes in TCR Vβ gene usage associated with acute dengue virus infection. To determine whether acute dengue virus infection causes significant shifts in the TCR Vβ gene usage in PBMCs, we examined the TCR Vβ gene usage of PBMCs obtained from each subject on study day 2, fever day +1 (one day after defervescence), and study day 180. For one patient in each group, only the study day 2 specimen was available for analysis. The mean interval between study day 2 and fever day +1 for the study population as a whole was 2.2 days (95% CI = 1.9–2.5), and was not significantly different between those children with DHF (mean = 2.2 days) and those with DF (mean = 2.3 days).
We did not note deletion of cells using any particular TCR Vß gene family following acute dengue virus infection in the study population. However, we detected TCR transcripts using Vß20 in only one of the 26 subjects studied, including those with primary dengue virus infection. We were able to amplify TCR Vß20 transcripts from PBMCs obtained from healthy donors from the United States and from six of the seven control Thai subjects using the same experimental procedures.

The results of our statistical analysis of the changes in TCR Vß gene usage for the study population as a whole are summarized in Table 2. All analyses for which a paired t-test showed $P < 0.05$ are shown; however, using our selected cutoff of $P < 0.01$, there were few TCR Vß gene families that showed significant changes in usage. From study day 2 to fever day +1 there was a statistically significant decrease in usage of Vß5.1. From fever day +1 to study day 180, there were statistically significant increases in usage of Vß2, Vß5.1, and Vß6 and statistically significant decreases in usage of Vß1 and Vß13.1. From study day 2 to study day 180, there was a statistically significant decrease in usage of Vß18.

Statistically significant changes in usage of one or more TCR Vß gene families during the acute infection were apparent in 12 subjects. The most common such finding was the expansion in use of a particular TCR Vß gene between study day 2 and fever day +1. We found expansion in use of a single TCR Vß gene family in six of 12 subjects with DHF and three of 12 subjects with DF ($P$ not significant). These expansions generally did not persist in the PBMCs obtained six months after the acute infection. For example, Figure 1A shows one subject (C94-050) with DHF and secondary dengue-4 virus infection who demonstrated increased use of Vß21 on fever day +1. Figure 1B shows one subject (C94-076) with DF and secondary dengue-1 virus infection who demonstrated increased use of Vß24 on fever day +1. The TCR Vß gene family showing the greatest increase in usage differed among the study subjects with such expansions, and we did not identify any consistent associations with the serotype of virus causing infection or the HLA alleles of the subject.

**Relationship of TCR Vß gene usage to disease severity.** We next compared the TCR Vß gene usage in the 13 subjects with DHF and the 13 subjects with DF (Figure 2). The TCR Vß gene usage at each of the three time points showed few differences between subjects with DHF and subjects with DF. Vß2 gene usage was significantly higher in subjects with DF on fever day +1 (3.8% versus 2.5%; $P = 0.006$). There was a trend toward higher Vß24 gene usage in subjects with DHF on study day 2 (3.3% versus 2.6%; $P = 0.016$), but this difference did not meet our criterion for significance. The results of our statistical analysis of the changes in TCR Vß gene usage in subjects with DHF and those with DF are summarized in Table 2. The change in Vß1 gene usage between study day 2 and study day 180 was significantly different between subjects with DHF and subjects with DF ($-1.3\%$ versus $+0.2\%; P = 0.010$). None of the other differences between the groups met our criterion for statistical significance.

**DISCUSSION**

We analyzed the TCR Vß gene usage of PBMCs obtained at three time points during and after acute dengue virus infec-
fection. The earliest time point studied (study day 2) corresponded to a mean fever day value of −1.2, or slightly more than one day before defervescence. This was the closest approximation available to the viremic phase of infection,\(^18\) and 24 of the 26 subjects studied had positive plasma virus culture at that time. The middle time point (fever day +1, one day after defervescence) corresponds closest to the period of plasma leakage in DHF.\(^17\) The latest time point (study day 180) is the closest approximation to a baseline for each subject since we did not have access to PBMCs from before the usage of 26 TCR V\(_\beta\) gene families. As shown in Figure 1, expansions in certain TCR V\(_\beta\) gene families showing expansion and the clinical, virologic, or genetic profile of the subjects. We found statistically significant changes in expression of TCR V\(_\beta\)1, V\(_\beta\)2, V\(_\beta\)5.1, V\(_\beta\)6, V\(_\beta\)13.1, and V\(_\beta\)18 in the study population as a whole. Although we used a relatively conservative criterion for statistical significance of \(P < 0.01\), these findings may still reflect type I error due to the large number of statistical comparisons performed. Therefore, we consider these to be preliminary observations that require confirmation in other study populations. We did not detect deletions of any specific TCR V\(_\beta\) gene families related to acute DV infection, regardless of the clinical severity of disease. This finding does not support the

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### Table 2

Summary of probability values for comparisons of interval changes in T cell receptor (TCR) gene usage in the study population\(^4\)

<table>
<thead>
<tr>
<th>V(_\beta)</th>
<th>Study day 2 to fever day +1</th>
<th>Fever day +1 to study day 100</th>
<th>Study day 2 to fever day +1</th>
<th>Fever day +1 to study day 180</th>
<th>Study day 2 to fever day +1</th>
<th>Fever day +1 to study day 180</th>
<th>Study day 2 to fever day +1</th>
<th>Fever day +1 to study day 180</th>
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<td>1</td>
<td>0.022</td>
<td>(↑) 0.001(^4)</td>
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<td>–</td>
<td>0.010</td>
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<td>5.1</td>
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<td>(↑) 0.002</td>
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<td>5.2</td>
<td>(↑) 0.005</td>
<td>(↑) 0.002</td>
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<td>6</td>
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\(^4\)All \(P\) values are 2-tailed. Dashes represent comparisons where \(P\) values are > 0.05. The direction of change in TCR gene usage is indicated only for comparisons showing \(P < 0.01\).

\(^5\)Logit-transformed values for TCR gene usage at the indicated time points were compared for all subjects combined by paired \(t\)-test.

\(^6\)Changes in logit-transformed values for TCR gene usage were compared between subjects with dengue hemorrhagic fever (DHF) and those with dengue fever (DF) by \(t\)-test.

\(^7\)(↑) or (↓), respectively, indicate an increase or decrease in V\(_\beta\) expression.

First, we looked for increases in the usage of some TCR V\(_\beta\) gene families in individual subjects during the acute infection, using the study day 180 specimen for comparison, as an indication of expansion of particular T cell populations. As shown in Figure 1, expansions in certain TCR V\(_\beta\) subsets during the course of DV infection were observed in some subjects. This finding is in agreement with our in vitro data demonstrating preferential expansion of V\(_\beta\)17-bearing T cells in the PBMCs of a dengue-immune donor upon stimulation with non-infectious DV antigen.\(^16\) The TCR V\(_\beta\) expansions were most often noted one day following defervescence (fever day +1), which supports the hypothesis that some of the atypical lymphocytes in the peripheral blood are proliferating DV-specific T cells.

The specific TCR V\(_\beta\) gene families showing expansion during acute DV infection differed among the study subjects. We did not identify any consistent association between the TCR V\(_\beta\) genes showing expansion and the clinical, virologic, or genetic profile of the subjects. We found statistically significant changes in expression of TCR V\(_\beta\)1, V\(_\beta\)2, V\(_\beta\)5.1, V\(_\beta\)6, V\(_\beta\)13.1, and V\(_\beta\)18 in the study population as a whole. Although we used a relatively conservative criterion for statistical significance of \(P < 0.01\), these findings may still reflect type I error due to the large number of statistical comparisons performed. Therefore, we consider these to be preliminary observations that require confirmation in other study populations.
FIGURE 1. T cell receptor (TCR) Vβ gene usage during and after acute dengue virus infection in individual subjects. Fever day +1 is one day after defervescence. A, subject C94-050 (dengue hemorrhagic fever [DHF]) had a significant expansion of Vβ21 gene usage on fever day +1. B, subject C94-076 (dengue fever [DF]) had a significant expansion of Vβ24 gene usage on fever day +1.
suggestion that a DV-encoded superantigen might be responsible for the increased T cell activation observed during DV infection, particularly in patients with DHF.\textsuperscript{7,8} Superantigens can induce marked activation followed by deletion of T cells expressing a particular TCR V\textbeta\textsuperscript{20} gene.\textsuperscript{24–26} Our failure to detect such an effect in this study population is subject to several limitations. Only one of the subjects studied had grade 3 DHF, and none of the cases of DHF were associated with a primary DV infection; massive activation of T cells not specific for classical DV antigens caused by interaction with a superantigen might be more likely to play a role in those clinical situations. Also, we detected TCR V\textbeta\textsuperscript{20} transcripts in only one of the 26 subjects. This may reflect genetic differences between the Thai and United States populations, but we cannot exclude the possibility that T cells using the V\textbeta\textsuperscript{20} gene were deleted earlier in infection or by a prior DV infection. Analysis of TCR V\textbeta\textsuperscript{20} gene usage in the PBMCs of DV-naive Thai children would distinguish between these possibilities.

Lastly, we compared the TCR V\textbeta\textsuperscript{20} gene usage in subjects with severe illness (DHF) with that in subjects with milder illness (DF). Although we found a significant difference in the usage of V\textbeta\textsuperscript{22} during the acute infection, the magnitude of this difference was small. Similarly, the statistically significant difference in the change in TCR V\textbeta\textsuperscript{1} gene usage from study day 2 to study day 180 between the two groups of subjects was small in magnitude. These differences may well reflect type I error. In general, our findings suggest that there are not qualitative differences in the T cell repertoire responding to acute DV infection between subjects with and
without plasma leakage, and that the increased T cell activation in DHF reflects quantitative differences in the T cell response.

Other groups have similarly failed to find differences between the TCR Vβ repertoire in the PBMCs of HIV-infected subjects and that of control subjects. However, skewing of the TCR Vβ repertoire has been observed in cells obtained from either the lungs14 or lymph nodes27 of HIV-infected patients. Others have also noted that alterations of TCR Vβ gene usage occur much less frequently in PBMCs compared to the organs or tissues in which disease pathology is directly observed.2,28 Dengue virus is believed to infect primarily monocytes and macrophages in vivo,30–32 and a specific organ or tissue that preferentially supports DV replication has not been identified. However it is possible that during acute DV infection, notable biases in Vβ expression may occur in tissues not examined in this study, such as lymph nodes or liver.

The pathogenesis of DHF is still poorly understood, but data suggest that increased levels of T cell activation play an important role. The cause of this heightened T cell activation is an active topic of investigation. To our knowledge, this study provides the first detailed analysis of TCR Vβ gene usage in the PBMCs of patients with acute DV infections. Our results provide additional insight into the nature and timing of T cell responses that should be helpful in directing future studies into the pathophysiological events in acute DV infection.

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