Characterization of T Cell Repertoire Changes in Acute Kawasaki Disease

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Summary

Kawasaki disease (KD) is an acute multisystem vasculitis of unknown etiology that is associated with marked activation of T cells and monocyte/macrophages. Using a quantitative polymerase chain reaction (PCR) technique, we recently found that the acute phase of KD is associated with the expansion of T cells expressing the V/32 and V/38.1 gene segments. In the present work, we used a newly developed anti-V/32 monoclonal antibody (mAb) and studied a new group of KD patients to extend our previous PCR results. Immunofluorescence analysis confirmed that V/32-bearing T cells are selectively increased in patients with acute KD. The increase occurred primarily in the CD4 T cell subset. The percentages of V/32 + T cells as determined by mAb reactivity and flow cytometry correlated linearly with V/3 expression as quantitated by PCR. However, T cells from acute KD patients appeared to express proportionately higher levels of V/32 transcripts per cell as compared with healthy controls or convalescent KD patients. Sequence analysis of T cell receptor/3 chain genes of V/32 and V/38.1 expressing T cells from acute KD patients showed extensive junctional region diversity. These data showing polyclonal expansion of V/32 + and V/38 + T cells in acute KD provide additional insight into the immunopathogenesis of this disease.

Kawasaki disease (KD) is an acute illness of early childhood characterized by fever, induration, and erythema of the hands and feet, inflammation of the mucous membranes, polymorphous skin rash, and cervical lymphadenopathy (1, 2). As a complication of this multisystem disease, coronary artery aneurysms or ectasia secondary to vasculitis develop in 15–25% of affected patients (3, 4). In the United States, KD is currently the major cause of acquired heart disease in children. Although epidemiologic evidence strongly supports an infectious etiology for this disease, the causative agent is unknown.

KD is associated with marked activation of T cells and monocyte/macrophages, as well as increased production of IL-1β, TNF-α, and IL-6 (for a review, see reference 5). The marked immune activation and production of cytokines are thought to play an important role in the pathogenesis of vascular endothelial cell injury during acute disease by eliciting proinflammatory and prothrombotic responses (6). These immunologic features are characteristic of diseases that are caused by microbial toxins that act as superantigens to stimulate a large proportion of T cells expressing specific TCR/3 chain variable (V/3) gene segments (7). In this regard, toxic shock syndrome, a disease with many clinical features similar to acute KD, is caused by staphylococcal toxic shock syndrome toxin 1 (TSST-1), a superantigen that induces the massive expansion, in vitro and in vivo, of V/32 + T cells (8).

Using a quantitative PCR technique, we recently found that the acute but not the convalescent phase of KD is associated with the expansion of T cells expressing V/32 and V/38 gene segments (9). In contrast, the T cell repertoire in patients with other febrile illnesses and exanthems did not undergo similar alterations (9). These observations suggested that the T cell activation associated with KD is due to superantigenic stimulation. An important characteristic of such
stimulation, however, is that the other variable elements (D, J, V, α, γ) of the TCR are not generally involved in antigen recognition (7). The present study was therefore carried out to characterize the TCR junctional diversity associated with the expansion of Vβ2 and Vβ8 T cells during acute KD and to use a newly available anti-Vβ2 mAb to extend our previous PCR studies.

Materials and Methods

Patients. 23 patients with KD (mean age, 38 mo; range, 10 mo–7 yr, 6 mo) were studied. The flow cytometry studies were done on 20 new KD patients, not previously reported on, whereas the cloning and sequencing of TCR β chain junctional regions were carried out on three Vβ2 amplified gene products obtained from three patients previously studied (9). The latter three samples were selected for their particularly high level of Vβ2 expression (see below). Acute KD was defined by the diagnostic guidelines of the American Heart Association (10) and required that patients have no clinical and/or laboratory evidence of any other disease known to mimic KD. Blood was drawn before intravenous gamma globulin treatment in 18 patients with acute KD (within 10 d of the onset of fever) and after 30 d of the onset of fever in 10 patients (convalescent KD). We were able to obtain eight paired samples from the child’s parents according to the guidelines of each medical center.

Lymphocyte Isolation and Culture. PBMC were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. PBMC were then analyzed either fresh or after culture with anti-CD3 and IL-2 to increase the production of functional TCR mRNA for the TCR Vβ analysis by PCR. In these experiments, PBMC were stimulated for 3 d at 37°C with anti-CD3 (20 ng/ml; Ortho Diagnostic Systems Inc., Westwood, MA), washed and expanded for 1 d in IL-2 as previously described (8, 9). Previous studies with cells from normal individuals have demonstrated that anti-CD3 stimulation for 3 d does not selectively expand a particular Vβ population compared with freshly isolated PBMC (8, 9).

Assessment of TCR Vβ Expression. We used two methods to assess the TCR Vβ repertoire in peripheral blood. The first method utilized two-color immunofluorescence with biotinylated mAbs directed at four different Vβ segments as previously described (11). These included mAbs directed to Vβ2 (clone E22E7; Immunotech, Marseille, France), Vβ3.1 (clone LC4; T Cell Science, Cambridge, MA), Vβ8.1,2 (clone MX6, [12]), and Vβ12 (clone S511, [13]), followed by incubation with streptavidin-PE (Fisher Biotech, Pittsburgh, PA) and FITC-conjugated anti-CD3, anti-CD4, or anti-CD8 (Olympus Corp., Lake Success, NY). The anti-Vβ2 antibody (murine IgM) was produced by using spleen cells from mice immunized with a murine TCR β chain-deficient hybridoma that had been transfected with a human-murine chimeric IVβ2-kDβ-hCβ-mβ DNA construct (Romagne, F., L. Besnardeau, and B. Malissen, manuscript in preparation). Its specificity was confirmed by showing that it stained T cell hybridomas expressing IVβ2, but not hybridomas expressing other human βs, as well as human T cell clones expressing Vβ2, but not human T cell clones expressing members of other Vβ families. The Vβ expression of the latter T cell hybridomas and clones were independently determined by PCR (14). In accord with quantitative PCR results (14), the blast population after in vitro stimulation with TSST-1 was greatly enriched for cells staining with E22E7 (>50% vs >10% staining in the starting population), whereas cells staining with this mAb were excluded from blasts after stimulation with staphylococcal enterotoxin B (SEB). Fluorescence intensity was analyzed with an Epics Profile Cytofluorograph (Coulter Electronics Inc., Hialeah, FL). Both freshly isolated and cultured PBMC were analyzed. Forward angle and 90° light-scatter patterns were used to gate on lymphocytes or blast cells in each case. The percentage of T cells bearing each TCR Vβ phenotype was expressed as a percentage of total T (CD3+) cells.

The second method utilized a quantitative PCR method to estimate the proportion of T cells expressing particular Vβ gene segments (14). In these experiments, 2 μg of total RNA prepared from anti-CD3-stimulated cells was used for the synthesis of first-strand cDNA. For each PCR, TCR β and α chain cDNAs were amplified using oligonucleotide primers specific for Vβ2 or Vβ12 and an oligomer from the downstream β chain C region (Cβ primer) as one pair, and two Ca primers as the other pair. The sequences of the specific primers used and details of the PCR have been published (14). The amount of product was quantitated by incorporation of 32P end-labeled 3′ primers added to the reactions (~106 cpm each). The data were expressed as amount of Vβ product over the amount of Ca product, or Vβ/Ca ratio, for each Vβ. The PCR technique employed 23 cycles of amplification. In separate experiments, we analyzed the quantitative nature of the PCR technique by examining input Vβ RNA versus amount of amplified PCR product obtained. Over the range of Vβ values obtained in this study, there was a direct linear correlation between the ratio of Vβ PCR product amplified and the amount of mRNA analyzed.

Cloning and Sequencing of TCR β Chain Junctional Regions. 10 μg of total RNA was used to synthesize cDNA followed by PCR with a Vβ2 or Vβ8.1 primer and a 3′ Cα primer. The sequences of the primers were 5′-GGGAATTCCATCAACCATTGCAAGCCTGACCT-3′ for Vβ2, 5′-GGGAATTCCATTATTTTACAAACAACGTCTCCG-3′ for Vβ8.1, and 5′-GGGAATTCATTTACTTTAACATGAGCTTGATGGCTGACCT-3′ for Vβ3. The Vβ and Cα primers carried EcoRI or BamHI sites, respectively (underlined). After 22 cycles of PCR, amplified β chain fragments were extracted with phenol-chloroform and precipitated with ethanol. Purified fragments were then digested with EcoRI and BamHI, ligated into plasmid pTZ18R (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and cloned. Double-stranded plasmid DNAs were prepared by alkali-lysis method and acid phenol extraction and were sequenced by Sequenase Ver2.0 kit (US Biochemical, Cleveland, OH) following the manufacturer’s recommendations (15).

Results and Discussion

In a previous study using a quantitative PCR technique and 22 different Vβ-specific primers, we reported markedly increased expression of Vβ2 and less so of Vβ8.1 gene segments by peripheral blood T cells from acute KD patients (9). A mAb to Vβ2 was not available at the time of those analyses, but has since been generated. In the following experiments, we analyzed TCR Vβ2 expression using a Vβ2-specific mAb and flow cytometry and compared these data to results obtained with the quantitative PCR technique on samples from a new group of KD patients. All cells in these experiments were initially stimulated with anti-CD3.
As shown in Fig. 1, the mean percentage of Vβ2+ T cells in 13 acute KD patients was significantly higher than the control group of 13 healthy children (mean ± SEM = 8.1 ± 0.5% for acute KD vs. 6.4 ± 0.3% for healthy children; p = 0.01 by two tailed unpaired Student's t test), or eight healthy adults (5.7 ± 0.5% for healthy adults; p = 0.007). In contrast, there was no significant difference seen in the percentages of T cells bearing Vβ5.1 between patients (2.9 ± 0.2%) and controls (2.6 ± 0.3%; p = 0.22). The mean percentage of Vβ2+ T cells in acute KD patients was also increased compared with convalescent KD patients (6.2 ± 0.6%; p = 0.03). A decrease in Vβ2+ T cells after the acute phase was also apparent in the eight patients followed serially (Fig. 1). Only one patient maintained an increased percentage of Vβ2+ T cells in convalescence. It is interesting that this patient developed mitral valve insufficiency as a complication of her KD. None of the other patients studied in convalescence showed signs of cardiac involvement.

We also analyzed the ratio of Vβ cDNA to Cα cDNA amplified by PCR using the same PBMC that were studied by immunofluorescence analysis. We analyzed Vβ2/Cα and Vβ12/Cα ratios of samples from both acute and convalescent KD patients and control subjects. To obtain a wide range for Vβ12 expression, some of the control PBMC were also stimulated with SEB (11) and analyzed by both immunofluorescence and PCR. The Vβ/Cα ratios obtained by PCR were compared with the percentages of T cells bearing a particular Vβ as determined by immunofluorescence. A linear correlation was found between these two methods of Vβ analysis (Fig. 2). In healthy controls and convalescent KD pa-

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**Figure 1.** Vβ2+ T cells are increased during the acute phase of Kawasaki disease. PBMC were stimulated with anti-CD3 and IL-2 and analyzed by immunofluorescence and flow cytometry with anti-Vβ2 and Vβ5.1 mAbs. Bars, mean ± SEM.

**Figure 2.** Correlation between Vβ expression as determined by immunofluorescence and by PCR. TCR Vβ expression in anti-CD3 activated T cells from patients with acute KD (●), convalescent KD (●), and healthy controls (△) as well as SEB-stimulated T cells from healthy controls (▲) was determined by both Vβ-specific mAbs and quantitative PCR. Cytofluorographic data are presented as the percentage of total cells staining positive, and PCR data are expressed as the ratio of the amount of labeled primers incorporated into the Vβ and Cα bands, i.e., the Vβ/Cα ratio. (Shaded areas) Mean ± 2 SD for both percentage of cells staining and Vβ/Cα ratios determined for a group of healthy controls. Results are shown for Vβ2 (left) and Vβ12 (right).

**Figure 3.** Freshly isolated PBMC from acute KD patients (filled bars) and healthy controls (open bars) were analyzed by two-color immunofluorescence with biotinylated anti-Vβ mAbs and FITC-conjugated anti-CD3, CD4, and CD8. Data represent the mean ± SEM.
### Table 1. Analysis of TCR β chain Junctional Sequences Containing Vβ2 or Vβ8.1 Gene Segments in T Cells from Acute KD Patients

| Donor | Junctional sequence | Junctional sequence |
|-------|---------------------|---------------------|
| KD3   | CSAR DREQGF NEKL (1.4) | CSAR DREQGF NEKL (1.4) |
| KD4   | CSAR DRTV NEKL (1.4) | CSAR DRTV NEKL (1.4) |
|       | CSAR GG NQPQH (1.5) | CSAR GG NQPQH (1.5) |
|       | CSAR RLG TGA NEQF (2.1) | CSAR RLG TGA NEQF (2.1) |
|       | CSAR ALAGD EQF (2.1) | CSAR ALAGD EQF (2.1) |
|       | CSAR SGR GRSY NEQF (2.1) | CSAR SGR GRSY NEQF (2.1) |
|       | CSAR QR SYNEQF (2.1) | CSAR QR SYNEQF (2.1) |
|       | CSAR LPEL WREGD TGE LF (2.2) | CSAR LPEL WREGD TGE LF (2.2) |
|       | CSAR PGA TGE LF (2.2) | CSAR PGA TGE LF (2.2) |
|       | CSAR DPR GELF (2.2) | CSAR DPR GELF (2.2) |
|       | CSAR SYPI STDTQY (2.3) | CSAR SYPI STDTQY (2.3) |
|       | CS RNTLGR TDTQY (2.3) | CS RNTLGR TDTQY (2.3) |
|       | CS KLAGAA DTQY (2.3) | CS KLAGAA DTQY (2.3) |
|       | CS VFDRE Y (2.4) | CS VFDRE Y (2.4) |
|       | CS ARGMQ NITY (2.4) | CS ARGMQ NITY (2.4) |
|       | CS TYAI KD EQY (2.7) | CS TYAI KD EQY (2.7) |
|       | CS SIGART YEQY (2.7) | CS SIGART YEQY (2.7) |
|       | CS TPSSGNL YEQY (2.7) | CS TPSSGNL YEQY (2.7) |
| KD3   | CASS PGTA EAF (1.1) | CASS PGTA EAF (1.1) |
| KD4   | CASS PGTA EAF (1.1) | CASS PGTA EAF (1.1) |
|       | CASS PTGN EAF (1.1) | CASS PTGN EAF (1.1) |
|       | CASS EDAS NYGYT (1.2) | CASS EDAS NYGYT (1.2) |
|       | CASS FGQQ NPQH (1.5) | CASS FGQQ NPQH (1.5) |
|       | CASS LGV QPQH (1.5) | CASS LGV QPQH (1.5) |
|       | CASS RPGD SPLH (1.6) | CASS RPGD SPLH (1.6) |
|       | CASS RTAGAF NEQF (2.1) | CASS RTAGAF NEQF (2.1) |
|       | CASS LAGGN NEQF (2.1) | CASS LAGGN NEQF (2.1) |
|       | CASS TSPGAT YNEQF (2.1) | CASS TSPGAT YNEQF (2.1) |
|       | CASS TWLG NEQF (2.1) | CASS TWLG NEQF (2.1) |
|       | CASS RDTGD GELF (2.2) | CASS RDTGD GELF (2.2) |
|       | CASS SFHEG EQY (2.5) | CASS SFHEG EQY (2.5) |
|       | CASS TSGG QY (2.7) | CASS TSGG QY (2.7) |
|       | CASS HK S EQY (2.7) | CASS HK S EQY (2.7) |
|       | CASS TQR KD YEQY (2.7) | CASS TQR KD YEQY (2.7) |

The Vβ2/Cα ratio was 0.26 for donor KD3, 0.29 for KD4, and 0.46 for donor KD5 as compared to a mean Vβ2/Cα ratio of 0.10 for normals. The Vβ8/Cα ratio was 0.14 for KD3 as compared to a mean Vβ8/Cα ratio of 0.07 for normals. Three clones that contained stop codons and two KD4 clones, one containing Jβ1.4 and the other Jβ2.7, that could not be defined in the junctional region, are not shown.

In patients, the slopes of the regression lines for Vβ2 and Vβ12 had similar values of 0.01 and 0.02, respectively, indicating that the Vβ fragments were amplified by PCR with similar efficiencies. It is interesting that in acute KD patients, a significantly steeper slope for Vβ2 (0.06; p = 0.003 by analysis of covariance) was seen. These data suggest that in addition to an increased percentage of Vβ2+ T cells, there was proportionately greater Vβ2 mRNA expressed per cell during acute KD. It is surprising that this increased expression per cell appears to be maintained despite in vitro activation with anti-CD3 and IL-2.

To determine whether the in vitro anti-CD3 activation had
an effect on the Vβ analyses, and to determine what T cell
dependent polyclonal expansion of T cells was observed.

In view of the selective expansion of Vβ2+ and Vβ8+ T
cells in acute KD (9), the extensive β chain junctional diver-
sity suggests that these Vβs play a dominant role in recogni-
tion. This type of recognition is not characteristic of responses
to most peptide antigens. A complex response to multiple
antigens or epitopes also seems unlikely since multiple Vβ
regions would be expected to be involved. A dominant role
for Vβ (vs. CDR3 region) has been occasionally observed
for T cell responses to particular peptide–MHC complexes
(17–19), including a human CD4+ T cell response to a
tetanus toxin-derived peptide being presented by different class
II MHC molecules (17). However, the apparent frequency of
responding cells in the circulation of acute KD patients
(>1 in 50) seems out-of-proportion to that expected for a
response to a conventional antigen. Together, this high
frequency of response and the prominent role for Vβ appear
to be more consistent with the hypothesis that T cell activa-
tion during the acute phase of KD is mediated by a superan-
tigen. Verification that KD is caused by a superantigen awaits
identification of the etiologic agent.

The mechanism by which marked T cell activation might
contribute to the clinical manifestations of KD is unclear.
Bacterial superantigens such as staphylococcal enterotoxins
and streptococcal erythrogenic toxins (SPEs) can cause mas-
sive T cell stimulation and cytokine release, including potent
induction of IL-1 and TNF. In recent studies of a murine
model of toxic shock syndrome, the in vivo release of TNF-α
was shown to be dependent on superantigen stimulation of
T cells (20, 21). As in toxic shock syndrome, T cell stimula-
tion may be critical to cytokine release in acute KD. The
production of increased circulating levels of IL-1 and TNF
as well as other cytokines, e.g., IL-6, have been reported to
occur during the acute phase of KD (5). These cytokines play
a critical role in the induction of inflammatory and throm-
botic responses (6). In the case of acute KD, the secretion
of IL-1 and TNF is associated with the expression of leuko-
cyte adhesion molecules which localize inflammatory cells
to the vascular wall and are thought to play an important
role in the pathogenesis of vasculitis in this disease (22).

The etiologic agent which causes KD is currently unknown.
Nevertheless, it is widely believed that KD is caused by an
infectious agent because of the acute self-limited nature of
this disease, geographical clustering of outbreaks, the clin-
ical symptoms of fever and exanthem mimicking other infec-
tious diseases, and the unique susceptibility of young chil-
dren, suggesting that humoral immunity to the KD agent
develops early in life (23). Our present results may provide
a new avenue of investigation into the potential etiology of
this fascinating disease.
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