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 Vβ2 and Vβ8.1 gene segments. In the present work, we used a newly developed anti-Vβ2 monoclonal antibody (mAb) and studied a new group of KD patients to extend our previous PCR results. Immunofluorescence analysis confirmed that Vβ2-bearing T cells are selectively increased in patients with acute KD. The increase occurred primarily in the CD4 T cell subset. The percentages of Vβ2$^+$ T cells as determined by mAb reactivity and flow cytometry correlated linearly with Vβ expression as quantitated by PCR. However, T cells from acute KD patients appeared to express proportionately higher levels of Vβ2 transcripts per cell as compared with healthy controls or convalescent KD patients. Sequence analysis of T cell receptor β chain genes of Vβ2 and Vβ8.1 expressing T cells from acute KD patients showed extensive junctional region diversity. These data showing polyclonal expansion of Vβ2$^+$ and Vβ8$^+$ T cells in acute KD provide additional insight into the immunopathogenesis of this disease.

Kawasaki disease (KD)[1] 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 β chain variable (Vβ) 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 Vβ2$^+$ 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 Vβ2 and Vβ8 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

[1] *Abbreviations used in this paper:* KD, Kawasaki disease; SEB, staphylococcal enterotoxin B; TSST-1, toxic shock syndrome toxin 1.

stimulation, however, is that the other variable elements (Dβ, Jβ, Vα, Jα) 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 acute and convalescent phase of KD. 13 children and 8 adults were studied as healthy controls. Because the highest prevalence of KD is seen in Japan, and 6 out of 23 of the KD patients in our study were Japanese, we included six Japanese children and four Japanese adults in the healthy control group although our previous study showed that ethnic differences did not affect the TCR repertoire (9). Informed consent was obtained from either the patient or 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), Vβ5.1 (clone LC4; T Cell Science, Cambridge, MA). Vβ8.1;8.2 (clone MX6, [12]), and Vβ12 (clone S511, [13]), followed by incubation with streptavidin-PE (1:100) (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 hVβ2-hDJβ-hCβ-mCβ 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 hVβ2, but not hybridomas expressing other human Vβs, 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 coamplified 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 Cα 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 ^{32}P end-labeled 3′ primers added to the reactions ($\sim 10^6$ cpm each). The data were expressed as amount of Vβ product over the amount of Cα product, or Vβ/Cα 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′ Cβ primer. The sequences of the primers were 5′-GGGAATTCTCATCAACCATGCAAGCCTGACCT-3′ for Vβ2, 5′-GGGAATTCATTTACTTTAACAACAACGTTCCG-3′ for Vβ8.1, and 5′-GGGATCCTTCTGATGGCTCAAACAC-3′ for Cβ. The Vβ and Cβ 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.

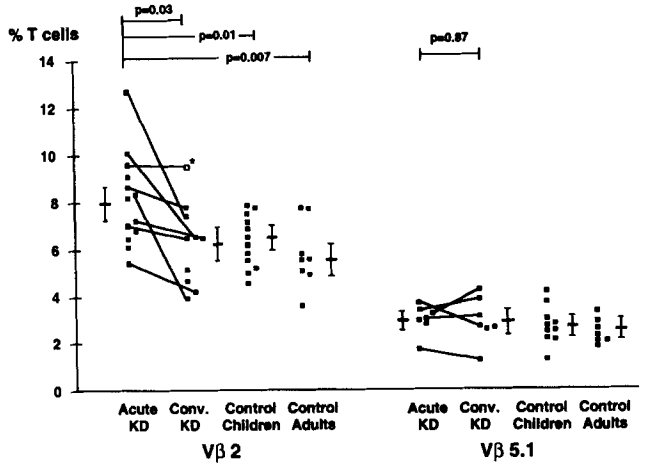

Figure 1. Vβ2⁺ T cells are increased during the acute phase of Kawasaki disease. PBMC were stimulated with anti-CD3 and IL-2 and analyzed by immunofluorescence and flow cytometry with anti-Vβ2 and Vβ5.1 mAbs. Bars, mean ± SEM.

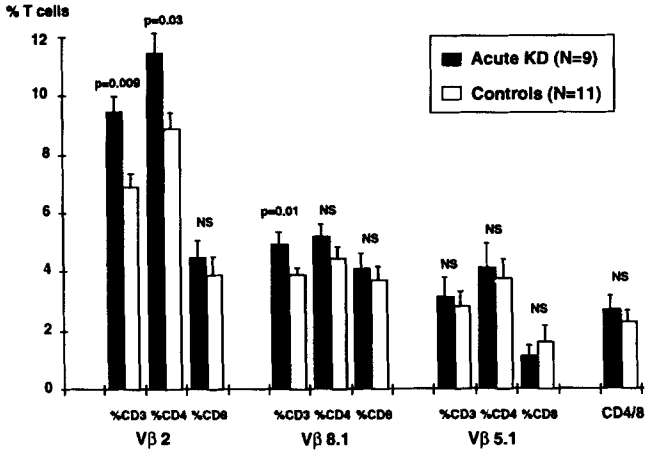

Figure 3. Freshly isolated PBMC from acute KD patients (*filled bars*) and healthy controls (*open bars*) were analyzed by two-color immunofluorescence with biotinylated anti-Vβ mAbs and FITC-conjugated anti-CD3, CD4, and CD8. Data represent the mean ± SEM.

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-

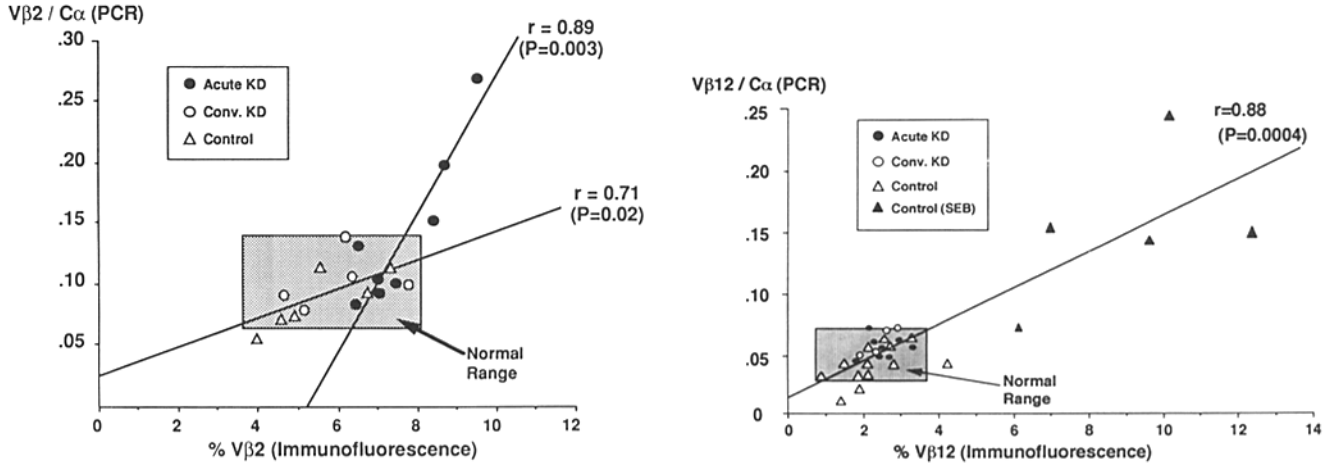

Figure 2. Correlation between Vβ expression as determined by immunofluorescence and by PCR. TCR Vβ expression in anti-CD3 activated T cells from patients with acute KD (●), convalescent KD (○), and healthy controls (△) as well as SEB-stimulated T cells from healthy controls (▲) was determined by both 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*).

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				Donor	Junctional sequence			
	Vβ	NDβN	Jβ			Vβ	NDβN	Jβ	
KD 3	CSAR	DREQGF	NEKL	(1.4)	KD5	CSA	RYRGLS	TEAF	(1.1)
(Vβ 2)	CSAR	DRTV	NEKL	(1.4)	(Vβ 2)	CSA	PSRDRGRK	TEAF	(1.1)
	CSAR	GG	NQPQH	(1.5)		CSA	YREM	NTEAF	(1.1)
	CSA	RLGTGA	NEQF	(2.1)		CSA	YRAMT	YGYT	(1.2)
	CSAR	ALAGD	EQF	(2.1)		CSA	PRDDLPQA	YGYT	(1.2)
	CSA	SGRLSY	NEQF	(2.1)		CSA	TDRVK	NTIY	(1.3)
	CSAR	QR	SYNEQF	(2.1)		CSA	GTGT	YNEQF	(2.1)
	CSA	LPELWREGD	TGELF	(2.2)		CSAR	HPRAY	EQF	(2.1)
	CSA	PGA	TGELF	(2.2)		CSA	SREGSS	ETQY	(2.5)
	CSAR	DPR	GELF	(2.2)		CSA	SGQV	QETQY	(2.5)
	CSA	SYPI	STDTQY	(2.3)		CSA	KKTGAL	ETQY	(2.5)
	CS	RNTLGR	TDTQY	(2.3)		CSA	KPGH	QETQY	(2.5)
	CSA	DRG	TDTQY	(2.3)		CSA	SQGGL	YEQY	(2.7)
	CSA	KLAGAA	DTQY	(2.3)		CSAR	DQTSRV	YEQY	(2.7)
	CS	VFDRE	Y	(2.4)		CSA	GLT	SYEQY	(2.7)
	CSA	ARGMQ	NIQY	(2.4)		CS	GIGT	SYEQY	(2.7)
	CSA	TYAGKD	EQY	(2.7)		CSAR	DVFGERVG	EQY	(2.7)
	CSA	SIGART	YEQY	(2.7)		CSAR	GAVGD	SYEQY	(2.7)
	CSA	TPSSGNL	YEQY	(2.7)					
KD4	CSAR	DVLSPS	EKL	(1.4)	KD 3	CASS	PGTA	EAF	(1.1)
(Vβ 2)	CSAR	DWGA	NQPQH	(1.5)	(Vβ 8)	CASS	PTGN	TEAF	(1.1)
	CSA	TSTGAGP	PLH	(1.6)		CAS	EDSAS	NYGYT	(1.2)
	CSA	RGGSDVRW	NEQF	(2.1)		CASS	FGQQ	NQPQH	(1.5)
	CSA	SGRAT	YNEQF	(2.1)		CASS	LVG	PQH	(1.5)
	CSA	SGF	YNEQF	(2.1)		CASS	PQRIGQA	QPQH	(1.5)
	CS	GRSWGGSN	NEQF	(2.1)		CAS	RPGD	SPLH	(1.6)
	CSA	GGLAG	TGELF	(2.2)		CAS	RTAGAF	NEQF	(2.1)
	CS	VPLWGPHWG	TDTQY	(2.3)		CASS	LAGGN	NEQF	(2.1)
	CSA	WTSGSS	TDTQY	(2.3)		CAS	TSPGAT	YNEQF	(2.1)
	CSAR	DTDK	DTQY	(2.3)		CAS	TWLGV	NEQF	(2.1)
	CSAR	GWTSGKG	ETQY	(2.5)		CASS	RDTGD	GELF	(2.2)
	CSAR	QY	QETQY	(2.5)		CAS	SFHEG	ETQY	(2.5)
	CSAR	WTSPFS	SYEQY	(2.7)		CASS	TSGG	QY	(2.7)
						CASS	HKS	EQY	(2.7)
						CASS	TQRKDE	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.

tients, 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 $V\beta2^+$ 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 subsets were expanded in KD, freshly isolated PBMC taken from nine patients with acute KD and 11 controls were assessed for expression of Vβ2, 5.1, or 8.1 on CD3, CD4, and CD8 T cells by direct two-color immunofluorescence and flow cytometry (Fig. 3). In the total T cell population, $V\beta2^+$ and $V\beta8.1^+$ cells were significantly increased in patients with acute KD ($p = 0.009$ and 0.01, respectively) but $V\beta5.1^+$ cells were not increased ($p = 0.9$). These data are consistent with the above data and our previous report (9). Although $V\beta2^+$ and $V\beta8.1^+$ T cells were increased in both CD4 and CD8 T cells in patients, Vβ2 expression in only the CD4 population was significantly different ($p = 0.03$) between patients and controls.

Superantigens stimulate T cells almost solely through the Vβ portion of the TCR and therefore induce an expansion of T cells mostly independent of their TCR junctional regions. This is markedly different from T cells activated by most conventional peptide antigens which frequently results in an expansion of only a subset of Vβ-expressing cells with limited TCR junctional diversity. To determine whether $V\beta2^+$ and $V\beta8^+$ T cells were expanded in a polyclonal vs. oligoclonal manner in acute KD, we analyzed the sequence of random cDNA clones containing PCR-amplified Vβ2 and Vβ8 segments (Table 1).

The sensitivity of our analysis for clonotypic expansion was initially determined by mixing normal PBMC with $V\beta8.1^+$ Jurkat cells. Analysis of TCR Vβ8.1 usage by flow cytometry showed that the addition of Jurkat cells increased the percentage of $CD3^+/V\beta8^+$ cells from 2.4% in the original PBMC sample to 4.8%. The Vβ8/Cα ratio assessed by PCR also increased from a baseline of 0.09 to 0.14, which was equal to the Vβ8/Cα ratio of acute KD sample no. 3 examined simultaneously (Table 1). Sequencing random cDNA clones containing PCR-amplified Vβ8.1 gene segments from the Jurkat-PBMC control mixture showed that 4 out of 10 cDNA clones (40%) encoded the same junctional sequence as Jurkat clones, proportional to the immunofluorescence data. These data indicated that our assay was sufficiently sensitive to detect oligoclonal expansions among samples with elevated Vβ/Cα ratios over the range of KD samples studied.

45 Vβ2 cDNA clones derived from three patients with acute KD who had a two- to threefold increase in Vβ2 gene expression, and 17 Vβ8.1 cDNA clones from a patient with a 60% increase in Vβ8 gene expression were sequenced. If acute KD were associated with a clonotypic expansion of $V\beta2^+$ and $V\beta8^+$ T cells, a significant percentage of the clones could have been expected to have identical junctional sequences. However, as shown in Table 1, none of the Vβ2 or Vβ8 clones had the same junctional sequence, and considerable diversity among the sequences within each individual was apparent. The CDR3 regions varied greatly in terms of residue number and sequence. The high proportion of charged residues in the junctional regions is similar to other T cell populations analyzed (16, 17), and a conserved motif is not apparent. In some sequence sets, preferential usage of certain Jβs (e.g., Jβ2.7 in patient KD5) may be suggested. However, this may represent a sampling artifact and similar results have been seen with normal samples (16).

In view of the selective expansion of $V\beta2^+$ and $V\beta8^+$ T cells in acute KD (9), the extensive β chain junctional diversity suggests that these Vβs play a dominant role in recognition. 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 activation during the acute phase of KD is mediated by a superantigen. 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 massive 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 stimulation 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 thrombotic responses (6). In the case of acute KD, the secretion of IL-1 and TNF is associated with the expression of leukocyte 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 clinical symptoms of fever and exanthem mimicking other infectious diseases, and the unique susceptibility of young children, 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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