T-Cell Receptor Vβ Gene Expression in Infiltrating Cells in Murine Hearts With Acute Myocarditis Caused by Coxsackievirus B3

Yoshinori Seko, MD; Hideo Yagita, PhD; Ko Okumura, MD; Yoshio Yazaki, MD

Background In viral myocarditis, we previously reported that natural killer cells infiltrate the heart first, then activated T cells infiltrate second and play an important role in the pathogenesis of the myocardial damage.

Methods and Results To elucidate the nature of T-cell infiltration, using a murine model of acute myocarditis caused by coxsackievirus B3, we analyzed the expression of T-cell receptor (TCR) Vβ genes in infiltrating cells in the heart by polymerase chain reaction (PCR). The PCR-amplified products were confirmed by Southern blot hybridization with a Cβ cDNA probe. In contrast to spleen lymphocytes, the repertoire of Vβ gene transcripts in the heart was restricted. The infiltrating cells expressing Vβ10 were found in six of eight hearts of mice with acute myocarditis. The infiltrating cells expressing Vβ8 and Vβ13 were found in four of eight hearts with myocarditis, respectively. Immunoperoxidase staining of serial sections of the heart of myocarditis for TCR αβ chains and TCR Vβ10 confirmed that the dominant population of infiltrating T cells expressed Vβ10 gene products.

Conclusions The restricted usage of TCR genes by infiltrating T-cells may indicate that a specific antigen in heart with myocarditis is targeted. Our findings raise the possibility of immunotherapy with monoclonal antibodies specific for TCR Vβ elements to prevent T-cell–mediated myocardial damage in viral myocarditis. (Circulation. 1994;89:2170-2175.)

Key Words • polymerase chain reaction • antigens • major histocompatibility complex

Viral myocarditis not only can cause congestive heart failure as an acute inflammatory disorder but also has been strongly implicated in the pathogenesis of idiopathic dilated cardiomyopathy. The pathogenesis of the myocardial cell damage involved is of great clinical significance and remains to be clarified. We previously reported that major histocompatibility complex (MHC) class I antigen is strongly induced on cardiac myocytes in murine acute myocarditis caused by coxsackievirus B3 (CVB3).1 We also demonstrated in the same model of acute myocarditis that natural killer (NK) cells expressing a cytolytic factor, perforin, infiltrate the heart. Infiltration by T-helper (Th) cells, then cytotoxic T lymphocytes (CTLs) subsequently occurs, suggesting that the expression of MHC class I antigen on cardiac myocytes facilitates the interaction between cardiac myocytes and CTLs and leads to further myocardial cell damage.2

In general, foreign antigens such as viruses are digested and degraded into peptide fragments in the target cell cytoplasm and then presented on the surface of the target cell membrane by MHC antigens. T cells specifically recognize processed antigens in conjunction with MHC molecules through their T-cell receptors (TCRs) that consist mostly of α and β chain heterodimers. The TCR α chain consists of variable, joining, and constant regions, while the β chain consists of variable, diversity, joining, and constant regions. They are designated as Vβ, Dβ, Jβ, and Cβ, respectively. The antigen specificity of the TCR is defined by the V domains encoded by variable, diversity, and joining gene elements that are rearranged and joined during T-cell differentiation. Recent studies on autoimmune diseases,3-5 allograft rejection,6 and malignancy7 have shown that the T cells involved in the local immune response use a limited range of TCR genes, indicating that they may interact with a specific antigen and might play an important role in the pathogenesis of these conditions.

The purpose of the present study was to clarify the immunologic mechanisms that may cause persistent damage to cardiac myocytes by investigating the pathophysiology of T-cell–mediated autoimmune processes in the later phase of myocarditis when the viral genome has almost disappeared. For this purpose, we analyzed the expression of TCR Vβ genes in infiltrating cells in the hearts of mice with acute viral myocarditis by using polymerase chain reaction (PCR). We also confirmed the expression of TCR Vβ gene products by immunoperoxidase staining.

Methods

Virus and Animals
Coxsackievirus B3 (Nancy strain) was a kind gift from Dr Y. Kitaura (Osaka Medical College, Osaka, Japan). It was grown in cultures of FL cells (human amnion), which were supplied by the Japanese Cancer Research Bank (JCRB)-Cell Bank (National Institute of Hygienic Sciences, Tokyo). The virus preparation had a titer of 1 × 10^9 plaque-forming units (PFU)/mL and was stored at −80°C. Seven-week-old C3H/He male mice were purchased from Shizuoka Laboratory Animal
Sequences of Primers Used for Polymerase Chain Reaction

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<th>Primer</th>
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Center (Shizuoka, Japan). They were inoculated intraperitoneally with 5 × 10⁴ PFU of CVB3 in 0.2 mL of phosphate-buffered saline (PBS). Five-week-old BALB/c male mice were also purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Preparation of RNA and cDNA Synthesis

Mice were killed on day 12 after virus inoculation, a time when T cells show significant increase among the cells infiltrating the heart. Total cytoplasmic RNA was prepared from the hearts and spleen tissues by a method using RNA zol (CINNA/BIOTEX Laboratories International, Inc.) according to the manufacturer’s instructions, and 0.5 to 1.0 μg of total RNA was used for the synthesis of single-stranded cDNA with reverse transcriptase. Briefly, in a volume of 20 μL 1×RTase buffer (40 mM/mL Tris-HCl, pH 8.3, 100 mM/L KCl, 10 mM/L MgCl₂, and 10 mM/L dithiothreitol), 1.5 mM/L deoxyribonucleotide triphosphates, 20 U of human ribonuclease inhibitor, 5 μM/mL 3'→5' primer, and 22 U of Rous-associated virus 2 (RAV-2) reverse transcriptase (TAKARA SHUZO Co, Ltd) were incubated with RNA (0.5 to 1.0 μg) for 60 minutes at 42°C. The reaction mixture was then denatured at 94°C for 5 minutes. Total cytoplasmic RNA was also prepared from the thymocytes of BALB/c mice and the peripheral blood lymphocytes (PBLs) of a C3H/He mouse on day 12 of CVB3-infection as controls.

Amplification of cDNA by PCR

Single-stranded cDNA from heart or spleen tissue was amplified using a 5'-Vβ-specific primer and a 3'-Cβ primer at a final concentration of 0.5 μM/mL each in the reaction mixture. Here we synthesized 20 different Vβ-specific oligonucleotides as 5'-sense primers and a Cβ-specific oligonucleotide as a 3'-antisense primer (Table). The sequences for primers were selected to have an equal length and nearly the same (G+C) contents to minimize differences in amplification. To discriminate homologous sequences (such as Vβ8.1, Vβ8.2, and Vβ8.3), primer sequences with different nucleotides at the 3' terminus were selected to achieve specific amplification. The nomenclature used for Vβ sequences was that defined by Wilson et al. Amplification was performed with 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in a DNA thermal cycler (Perkin-Elmer Cetus). The PCR was performed with 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 2 minutes, and primer extension at 72°C for 3 minutes.

Southern Blot Analysis

Ten microliters of Vβ-Cβ-amplified products was subjected to electrophoresis on 2% agarose gel and transferred to a nylon membrane. Filters were prehybridized for 2 hours at 65°C in 6× SSPE, 2× Denhardt’s solution, 0.5% SDS, and 100 μg/mL salmon sperm DNA and hybridized overnight at 65°C in the same solution with a 32P-labeled BamHI/EcoRI fragment of the 86T1 gene, which mainly consists of the Cβ region. The filters were subsequently washed in 0.1× SSPE and 0.1% SDS for 1 hour at 65°C and then were autoradiographed.

Monoclonal Antibodies

A hamster anti-mouse TCR αβ monoclonal antibody (mAb) (hybridoma H57-597) and a rat anti-mouse Vβ10 mAb (B21.5) were purchased from PharMingen.

Immunohistochemistry

Mice were killed on day 12 after virus inoculation. Cryostat sections (6 μm thick) of heart ventricles were prepared, air dried, and fixed in acetone for 5 minutes at 4°C. After washing in PBS, the serial sections were incubated with hamster anti-mouse TCR αβ mAb and rat anti-mouse TCR Vβ10 mAb, respectively, for 1 hour at 37°C. After washing in PBS, the sections were incubated with biotinylated goat anti-hamster IgG antibody (Caltag Laboratories, Inc) for anti-TCR αβ mAb, biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Inc) for anti-TCR Vβ10 mAb for 1 hour at 37°C. After washing in PBS, the sections were incubated with avidin-biotinylated peroxidase complex (ABC-immunoperoxidase kit, Vector Laboratories, according to the manufacturer’s instructions) for 30 minutes at 37°C, washed in PBS, and reacted with diaminobenzidine tetrahydrochloride (0.2 mg/mL). After washing in PBS, the sections were counterstained with hematoxylin and then dehydrated in ethanol, and the coverslips were mounted in xylene with resin.

Results

Expression of TCR Vβ Genes in Thymocytes and PBLs

To examine the efficiency of the Vβ primers, we analyzed the expression of TCR Vβ genes in PBLs of a CVB3-infected C3H/He mouse as well as BALB/c mouse thymocytes because the BALB/c mouse is known to express almost all of the Vβ gene family. Fig 1 shows the results of Southern blot analysis. There was expression of all Vβ genes in BALB/c mouse thymocytes. In PBLs of a CVB3-infected C3H/He mouse, there was also expression of almost all Vβ genes; however, several Vβ genes (especially Vβ17a) were only weakly expressed. The absence of these transcripts may have reflected clonal elimination induced by the negative selection process that occurs in the thymus. These results indicate that the PCR using the Vβ and the Cβ primers listed in the Table could amplify the individual Vβ gene transcripts. We also examined the expression of Vβ genes in splenic lymphocytes of C3H/He mice.
without CVB3 infection. There was diverse and heterogeneous expression of Vβ genes in splenic lymphocytes (spleen) of a noninfected C3H/He mouse (Fig 1).

**Restricted Vβ Gene Expression in Infiltrating Cells in the Heart With Acute Viral Myocarditis**

To investigate TCR Vβ usage at the site of inflammation, Vβ transcripts in infiltrating cells in the heart of CVB3-infected mice were amplified by the PCR method. We also investigated TCR Vβ usage in the splenic lymphocytes of each mouse as a control, because it was difficult to prepare a sufficient amount of RNA from PBLs of each mouse. Fig 2 shows the results of Southern blot analysis of the PCR-amplified products obtained from splenic lymphocytes (Fig 2, spleen) and infiltrating cells in the heart (Fig 2, heart) of eight mice. There was diverse and heterogeneous expression of Vβ genes in splenic lymphocytes of all eight mice. Although some of the Vβ genes were not expressed, the Vβ gene usage was not restricted. As shown in Figs 1 and 2, we found that the Vβ gene usage in splenic lymphocytes of noninfected mice was similar to that in infected mice. In contrast, only a few Vβ genes were preferentially rearranged and transcribed in infiltrating cells in the heart with acute viral myocarditis. TCR Vβ10 was rearranged in six of eight mice (mouse 1, 2, 3, 4, 5, and 6); TCR Vβ8 families were rearranged in four of eight mice (mouse 1, 2, 5, and 8); TCR Vβ13 was rearranged in four of eight mice (mouse 3, 4, 6, and 7). Thus, the Vβ gene usage in infiltrating cells in the heart was restricted and the Vβ10 transcript was one of the dominantly expressed Vβ genes among the eight mice.

**Predominant Expression of TCR Vβ10 Gene Product in the Infiltrating Cells**

To confirm that the infiltrating T cells actually expressed a restricted repertoire of TCR Vβ gene products, especially Vβ10, we performed immunoperoxidase staining of serial sections of the ventricular tissue for TCR aβ chain and that of Vβ10 on day 12 after virus inoculation. Fig 3 shows the results of immunoperoxidase staining of serial sections of the ventricular tissue for TCR aβ chains (Fig 3A) and Vβ10 (Fig 3B). Comparison of the distribution of infiltrating T cells positive for TCR aβ chains and that of Vβ10 confirmed that the dominant population of infiltrating T cells expressed the Vβ10 gene product in this mouse.

**Discussion**

In this study, we demonstrated that TCR Vβ gene usage by infiltrating cells in acute murine myocarditis was restricted and that Vβ10 gene transcript and gene product was one of the predominantly expressed in infiltrating T lymphocytes, suggesting the possible role of Vβ10+ T lymphocytes in triggering persistent myocardial damage in the a later phase of viral myocarditis. We also found that Vβ8 and Vβ13 expressing T cells participated in the immune response.

Restricted TCR gene expression has been reported in several autoimmune diseases, and one of the best characterized models is murine experimental autoimmune...
mune encephalomyelitis, which is an animal model of human multiple sclerosis. Acha-Orbea et al.\(^2\) and Urban et al.\(^3\) demonstrated that myelin basic protein-specific T-cell clones predominantly used TCR V\(\beta\)8, and that in vivo administration of an anti-V\(\beta\)8 antibody significantly suppressed the induction of this autoimmune disease. Oksenberg et al.\(^4\) reported the restricted expression of TCR V\(\alpha\) genes in infiltrating T cells in the brain of multiple sclerosis patients. In the case of rheumatoid arthritis, Sottini et al.\(^5\) reported the restricted expression of TCR V\(\beta\) but not V\(\alpha\) genes in synovial fluid T cells, suggesting that a superantigen that selectively interacts with a TCR V\(\beta\) component may be associated with the pathogenesis of this disease. Other studies\(^6\) demonstrated the predominant expression of TCR V\(\beta\)2 and V\(\beta\)13 genes in infiltrating T cells in lips of Sjögren’s syndrome patients and also predominant expression of TCR V\(\beta\)11 genes in infiltrating T cells in pancreases of young nonobese diabetic mice, a model of insulin-dependent diabetes mellitus. Restricted expression of TCR genes also has been reported in tumor-infiltrating cells, indicating that a specific antigen in the malignant tumors is targeted.\(^7\) Further studies of TCR gene expression at primary and metastatic sites have revealed different TCR gene usage in tumor-infiltrating lymphocytes within the metastases of various organs, suggesting that there are the differences in tumor antigenicity at these sites.\(^8\) In the case of virus infection, it is known that selective depletion of T cells bearing specific TCR V\(\beta\) repertoire occurs during retrovirus infection such as human immunodeficiency virus and mouse mammary tumor virus.\(^9\) This may indicate the presence of superantigens encoded by the retroviruses. To our knowledge, there have been no reports demonstrating

the restricted TCR gene usage in infiltrating T cells at the site of inflammation in viral infection, especially viral myocarditis. In the present study, we demonstrated for the first time the restricted TCR V\(\beta\) gene expression in murine viral myocarditis. The results of our previous studies\(^10\) suggested that CTLs infiltrate the heart in the later phase of acute viral myocarditis and recognize a virus-derived antigen or some other antigen in conjunction with MHC class I antigen and may cause further myocardial damage. Most antigens are presented at the groove of the MHC molecule as a processed peptide fragment and are specifically recognized by T cells through the V domains of both the TCR \(\alpha\) and \(\beta\) chains. However, another category of antigens, the superantigens, are recognized through TCR V\(\beta\) region alone, independently of the V\(\alpha\) region. To approach the characteristics of the antigen newly expressed on cardiac myocytes after viral infection, using the same model of viral myocarditis, we are currently investigating the expression of TCR V\(\alpha\) genes in infiltrating cells in the heart.

Our findings suggest the possibility of using antibodies specific for the TCR V\(\beta\) gene products to prevent T-cell-mediated myocardial damage in the late phase of myocarditis when the viral genome has almost disappeared. Vanderbank et al.\(^11\) reported that immunization with a synthetic TCR V region peptide completely prevented the induction of experimental autoimmune encephalomyelitis in rats and suggested that the induction of anti-idiotypic antibodies against TCR V-region determinants played a crucial role in this process. Thus, TCR V-region peptide vaccination may be a useful form of immunotherapy for various autoimmune diseases characterized by restricted TCR V-gene usage as well as

![Fig 2. Southern blot analysis of the expression of T-cell receptor V\(\beta\) gene segments in splenic lymphocytes (spleen) and infiltrating cells in the heart (heart) from eight C3H/He mice infected with coxsackievirus B3. In both samples, the polymerase chain reaction was performed using each of the 20 T-cell receptor V\(\beta\) family-specific primers and a common C\(\beta\) primer. Amplified DNAs were hybridized with \(^{32}\)P-labeled C\(\beta\) probe.](http://circ.ahajournals.org/lookup/doi/10.1161/CIRCULATIONAHA.107.700853)
viral myocarditis. This approach would seem to have certain advantages over the in vivo administration of antibodies, which consisted of animal immunoglobulin. However, the present study was conducted in an inbred strain of mice; different strains of mice may show different responses, and extrapolation of the findings to the outbred human population will require further investigation.

Acknowledgments

This work was supported by a grant for cardiomyopathy from the Ministry of Health and Welfare, Japan, Japan Heart Foundation Research Grant For 1990, a grant from Yamakouchi Foundation for Research on Metabolic Disorders, and a grant from Japan Foundation for Health Sciences.

References


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Circulation. 1994;89:2170-2175
doi: 10.1161/01.CIR.89.5.2170

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