Restricted Usage of T Cell Receptor Vα-Vβ Genes in Infiltrating Cells in the Hearts of Patients with Acute Myocarditis and Dilated Cardiomyopathy

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Abstract

Prolonged myocardial cell damage initiated by acute myocarditis is thought to be one of the most important etiology of dilated cardiomyopathy. To investigate the immunological mechanisms involved in the pathogenesis of dilated cardiomyopathy, we analyzed the phenotypes of infiltrating cells and examined the expression of perforin in infiltrating cells in the hearts of patients with dilated cardiomyopathy as well as acute myocarditis. We also examined the expression of HLA and intercellular adhesion molecule-1 (ICAM-1) in myocardial tissue of these patients. Furthermore, to evaluate the antigen specificity of infiltrating T cells and persistence of viral genomes in the myocardial tissue, we analyzed the expression of T cell receptor (TCR) Vα and Vβ genes as well as enterovirus genomes by PCR. We found infiltration of perforin-expressing killer cells and enhanced expression of HLA class I and ICAM-1 in the myocardial tissue. We also found that the repertoires of TCR Vα as well as Vβ gene transcripts were restricted, indicating that a specific antigen in the hearts was targeted. Because no enterovirus genomes were detected in all patients, it is strongly suggested that a cell-mediated autoimmune mechanism triggered by virus infection may play a critical role in the pathogenesis of dilated cardiomyopathy. However, we could not exclude the possibility that viruses other than enteroviruses could be pathogenic in these patients. (*J. Clin. Invest.* 1995. 96:1035–1041.) Key words: perforin • HLA • intercellular adhesion molecule-1 • virus genome • PCR

Introduction

Patients with viral myocarditis usually present with acute heart failure, and the majority of them are thought to improve within several weeks. However, it is known that some of the patients go on to develop prolonged heart failure and clinical features resembling those of dilated cardiomyopathy in the later course of the disease. On the other hand, with use of endomyocardial biopsy, histologic evidence of active inflammation was reported to exist in 12–70% of patients with dilated cardiomyopathy (1–4). These strongly suggest that the persistent myocardial cell damage involved in viral myocarditis may cause continuous destruction of contractile proteins and facilitate fibrosis, which may finally lead to dilated cardiomyopathy. Until now, many studies (5–7) using murine models of viral myocarditis have shown that cell-mediated autoimmunity plays an important role in the pathogenesis of the observed myocardial cell damage. We (8, 9) previously reported that MHC class I antigen and intercellular adhesion molecule-1 (ICAM-1)[1] were strongly induced on cardiac myocytes in murine acute myocarditis caused by coxsackievirus B3 (CVB3). We (10) 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 and ICAM-1 on cardiac myocytes facilitates the interaction between cardiac myocytes and CTLs and leads to further myocardial cell damage.

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 Vα, Jα, Cα or 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 which are rearranged and joined during T cell differentiation. Studies on autoimmune diseases (11–13), allograft rejection (14), and malignancy (15) showed 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. Recently, we have demonstrated that a limited number of TCR Vβ genes were expressed and rearranged in infiltrating cells in the heart of acute murine viral myocarditis (16).

The purpose of the present study was to clarify the immunological mechanisms which may cause persistent damage to car-

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1. *Abbreviations used in this paper:* ICAM-1, intercellular adhesion molecule-1; CVB3, coxsackievirus B3; Th cells, T-helper cells; CTLs, cytotoxic T lymphocytes; NK cells, natural killer cells; TCR, T cell receptor.

diac myocytes and play a critical role in the pathogenesis of dilated cardiomyopathy. For this purpose, first, we analyzed the phenotypes of infiltrating cells and expression of perforin in infiltrating cells in the hearts of patients with dilated cardiomyopathy as well as acute myocarditis. We also analyzed the expression of HLA and ICAM-1 in myocardial tissue of these patients. Second, to examine the antigen specificity of infiltrating T cells, we analyzed the expression of TCR Vα and Vβ genes by PCR. Third, to examine whether or not virus genomes still exist in the myocardial tissues of these patients, we analyzed the expression of enterovirus genomes by a PCR method.

We found infiltration of perforin-expressing killer cells and enhanced expression of HLA class I and ICAM-1 in myocardial tissue of patients with dilated cardiomyopathy as well as acute myocarditis. Furthermore, the repertoires of TCR Vα as well as Vβ gene transcripts in the hearts of these patients were restricted, indicating that a specific antigen in the hearts was targeted.

Methods

Patients. Myocardial tissue samples were obtained at autopsy from two patients with acute myocarditis and four patients with dilated cardiomyopathy (five male and one female, average age = 41.3 yr), in whom clinical diagnoses of acute myocarditis and dilated cardiomyopathy had been previously determined by history, physical examination, blood analyses, and echocardiography. Four patients with dilated cardiomyopathy had congestive heart failure of 2 mo, 3 yr, and 10 yr (two patients) duration, respectively.

Virus and animals. CVB3 (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 1×10^8 plaque-forming units (PFU)/ml and was stored at −80°C. 7-wk-old C3H/He male mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were inoculated intraperitoneally with 5×10^6 PFU of CVB3 in 0.2 ml of PBS.

mAbs. Mouse anti-human CD4 (hybridoma Leu-3a), CD8 (Leu-2a), CD14 (Leu-M3), and CD20 (Leu-16) mAbs were purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Mouse anti-human CD16 (3G8) and TCR $\gamma\delta$ (Identi-T TCR δ1) mAbs were purchased from Immunotech (Marseille, Cedex, France) and T Cell Sciences, Inc. (Cambridge, MA), respectively. The procedures for preparing a rat anti-mouse perforin (P1-8) mAb, which was also shown to react with human perforin, have been previously described (17).

Mouse anti-HLA class I (W6/32) was purchased from Dakopatts (Glostrup, Denmark), and a mouse anti-human ICAM-1 (RR1/1) mAb was a gift from Dr. T. A. Springer (Harvard Medical School, MA).

Immunoperoxidase. Freshly dissected myocardial tissue samples were frozen in liquid nitrogen. Cryostat sections (6-μm thick) were prepared, air dried, fixed in acetone for 5 min, and incubated with mouse anti-human CD4, CD8, CD14, CD16, CD20, TCR $\gamma\delta$, HLA class I, ICAM-1 mAbs for 1 h at 37°C. For the staining of perforin, cryostat sections were fixed in acetone for 3 min, washed in PBS and then in 4% paraformaldehyde in PBS for 1 min. After washing in PBS, the sections were treated with 0.5% periodic acid for 10 min, washed in PBS, then incubated with rat anti-perforin mAb for 1 h at 37°C. After washing in PBS, the sections were incubated with biotinylated anti-mouse IgG or anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA) for 1 h at 37°C. After washing in PBS, the sections were incubated with avidin-biotinylated immunoperoxidase complex (ABC-Immunoperoxidase Kit, Vector Laboratories, according to the manufacturer's instructions) for 30 min at 37°C and washed in PBS, followed by reaction with diaminobenzidine tetrahydrochloride (0.2 mg/ml). After washing in PBS, the sections were counterstained with hematoxylin, dehydrated in ethanol, and the coverslips were mounted in xylene with resin.

Table I. Sequences of Primers Used for PCR

Primer	5′ → 3′ sequence	Family members
Vα1	TTGCCCTGAGAGATGCCAGAG	1.1–3
Vα2	GTGTTCCAGAGGGAGCCATTGCC	2.1–2
Vα3	GGTGAACAGTCAACAGGGAGA	3.1
Vα4	ACAAGCATTACTGTACTCCTA	4.1
Vα5	GGCCCTGAACATTCAGGA	5.1
Vα6	GTCACTTTCTAGCCTGCTGA	6.1
Vα7	AGGACCATTGTCCAGATAAA	7.1–2
Vα8	GGAGAGAATGTGGAGCAGCATC	8.1–2
Vα9	ATCTCAGTGCTTGTGATAATA	9.1
Vα10	ACCCAGCTGTGGAGCAGAGCCCT	10.1
Vα11	AGAAAGCAAGGACCAAGTGTT	11.1
Vα12	CAGAAGGTAACTCAAGCGCAGACT	12.1
Vα13	GCTTATGAGAACACTGCGT	13.1
Vα14	GCAGCTTCCCTTCCAGCAAT	14.1
Vα15	AGAACCTGACTGCCCAGGAA	15.1
Vα16	CATCTCCATGGACTCATATGA	16.1
Vα17	GACTATACTAACAGCATGT	17.1
Vα18	TGTCAGGCAATGACAAG	18.1
5′-Cα	GAACCCTGACCCTGCCGTGTACC	
3′-Cα	ATGTCTAGCACAGTTTTGTCTGTG	

Preparation of RNA and cDNA synthesis. Total cytoplasmic RNA was prepared from the freshly dissected myocardial tissue samples by a method using RNA zol (CINNA/BIOTECX Laboratories International, Inc., Friendswood, TX, according to the manufacturer's instructions), and 10–20 μg of total RNA was used for the synthesis of single-stranded cDNA with reverse transcriptase. Briefly, in a volume of 40 μl 1× RTase buffer (50 mM Tris-HCl, pH8.3, 75 mM KCl, 3 mM $MgCl_2$), and 10 mM dithiothreitol, 0.5 mM deoxyribonucleotide triphosphates, 25 μg/ml oligo $d(T)^{12-18}$, and 400 U of Moloney Murine Leukemia Virus (M-MLV H RT [Superscript]) reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) were incubated with RNA (10–20 μg) for 50 min at 42°C. The reaction mixture was denatured at 90°C for 5 min, then treated with RNase H (GIBCO-BRL) for 20 min at 37°C. Total cytoplasmic RNA was also prepared from the peripheral blood lymphocytes (PBLs) of a normal subject as controls.

For the detection of enterovirus genomes in the myocardial tissue, we also prepared total cytoplasmic RNA from the myocardial tissue of C3H/He mice on day 7 of CVB3-infection as a positive control.

Amplification of cDNA by PCR. Single-stranded cDNA from myocardial tissues or PBLs was amplified using a 5′-Vα or 5′-Vβ-specific primer and a 3′-Cα or 3′-Cβ primer at a final concentration of 0.5 μM each in the reaction mixture. We synthesized 18 different Vα-specific oligonucleotides and a Cα-specific oligonucleotide as 5′-sense primers, and another Cα-specific oligonucleotide as a 3′-antisense primer. We also synthesized 22 different Vβ-specific oligonucleotides and a Cβ-specific oligonucleotide as 5′-sense primers, and another Cβ-specific oligonucleotide as a 3′-antisense primer. The sequences for primers were selected as previously reported (15, 20, 21) (Tables I and II). Amplification was performed with 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a DNA thermal cycler (Perkin-Elmer Cetus). The PCR was performed with 30 or 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and primer extension at 72°C for 3 min.

For the detection of enterovirus genomes in the myocardial tissue, we also performed PCR gene amplification (30 cycles) using a 5′-sense primer (5′-TCCTCCGGCCCCTGAATGCG-3′) and a 3′-antisense

Table II. Sequences of Primers Used for PCR

Primer	5′ → 3′ sequence	Family members
Vβ1	GCACAACAGTTCCCTGACTTGCAC	1.1–2
Vβ2	TCATCAACCATGCAAGCCTGACCT	2.1–3
Vβ3	GTCTCTAGAGAGAAGAAGGAGCGC	3.1–2
Vβ4	ACATATGAGAGTGGATTTGTCATT	4.1–3
Vβ5.1	ATACTTCAGTGAGACACAGAGAAAC	5.1
Vβ5.2–3	TTCCCTAACTATAGCTCTGAGCTG	5.2–3
Vβ6	AGGCCTGAGGGATCCGTCTC	6.1–3
Vβ7	CCTGAATGCCCCAACAGCTCTC	7.1–2
Vβ8	ATTTACTTTAACAACAACGTTCCG	8.1–4
Vβ9	CCTAAATCTCCAGACAAAGCTCAC	9.1
Vβ10	CTCCAAAAACTCATCCTGTACCTT	10.1–2
Vβ11	TCAACAGTCTCCAGAATAAGGACG	11.1–2
Vβ12	AAAGGAGAAGTCTCAGAT	12.1–2
Vβ13.1	CAAGGAGAAGTCCCCAAT	13.1
Vβ13.2	GGTGAGGGTACAACTGCC	13.2
Vβ14	GTCTCTCGAAAAGAGAAGAGGAAT	14.1
Vβ15	AGTGTCTCTCGACAGGCACAGGCT	15.1
Vβ16	AAAGAGTCTAAACAGGATGAGTCC	16.1
Vβ17	CAGATAGTAAATGACTTTCAG	17.1
Vβ18	GATGAGTCAGGAATGCCAAAGGAA	18.1
Vβ19	CAATGCCCCAAGAACGCACCCTGC	19.1
Vβ20	AGCTCTGAGGTGCCCCAGAATCTC	20.1
5′-Cβ	CCCGAGGTCGCTGTGTTTGAGCCATCAGAA	
3′-Cβ	AGGCGGCTGCTCAGGCAGTATCTGGAGTCA	

primer (3′-ACCGACGAATACCACTGTTA-5′), which were shown to allow the detection of enterovirus genomes specifically (18).

Southern blot analysis. 10 μl of Vα-Cα or Vβ-Cβ amplified products were subjected to electrophoresis on 2% agarose gel and transferred to a nylon membrane. We used 5′-Cα or 5′-Cβ primer as probes for the detection of Vα-Cα or Vβ-Cβ amplified products, respectively. 5′-Cα and 5′-Cβ primers were labeled by terminal deoxynucleotidyl transferase at the 3′ end with phosphorus-32-α-dCTP. Filters were prehybridized for 2 h at 55°C in (6× SSPE, 2× Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA) and hybridized overnight at 55°C in the same solution with a ^{32}P-labeled 5′-Cα or 5′-Cβ primer. The filters were subsequently washed in (0.1× SSPE and 0.1% SDS) for 1 h at 55°C, and then were autoradiographed.

For the detection of PCR-amplified enterovirus genomes, we used a full-length CVB3 cDNA clone (pCB3-M1) (19), which was a generous gift from Dr. R. Kandolf (Max Planck Institute for Biochemistry, Martinsried, Germany), as a probe. The CVB3 cDNA was labeled by Klenow enzyme with ^{32}P-α-dCTP and an unlabeled mixture of dATP, dGTP, and dTTP. The following procedures for Southern blot analysis were the same as those for TCR samples.

Results

Histological examination showed that there were massive cell infiltration, extensive myocyte necrosis and degeneration with occasional irregular hypertrophy of myocytes in the left ventricle with acute myocarditis (cases 1 and 2). There were mild to moderate cell infiltration, widespread myocyte degeneration and necrosis with moderate interstitial fibrosis in the ventricles with dilated cardiomyopathy (cases 3–6). On the other hand, there were massive cell infiltration, marked myocyte necrosis and degeneration with occasional irregular hypertrophy of myocytes in the right atrium of a case with dilated cardiomyopathy (case 4).

Phenotypic analysis of the infiltrating cells. The relative distribution of phenotypic markers among the infiltrating cells in the heart of each patient are summarized in Table III. As shown in Table III, most of the infiltrating cells in the hearts of these patients consisted of CD16$^+$ NK cells, CD14$^+$ macrophages, CD8$^+$ CTLs, and CD4$^+$ Th-cells. There were only few infiltrating γδ T lymphocytes positive for TCR γδ and almost no infiltrating B-cells positive for CD20. Although we could study only two patients with acute myocarditis, heart tissue from acute myocarditis (cases 1 and 2) had a higher percentage of CTLs as compared with dilated cardiomyopathy.

Expression of perforin in infiltrating cells and HLA class I as well as ICAM-1 in myocardial cells in the hearts with acute myocarditis and dilated cardiomyopathy. Next, to investigate whether these infiltrating cells were actively involved in the myocardial damage, we examined the expression of a cytolytic factor, perforin in the infiltrating cells. We also examined the expression of HLA class I as well as ICAM-1 in the myocardial cells, because these antigens are known to play an important role in the interaction between target cells and killer lymphocytes such as CTLs (8, 9). Fig. 1 shows the expression of perforin in infiltrating cells and HLA class I as well as ICAM-1 in myocardial cells in the hearts with acute myocarditis (case 1) and dilated cardiomyopathy (cases 3–5). In all cases, there was clear expression of perforin in the cytoplasmic granules of some of the infiltrating cells (Fig. 1, *A, D, G,* and *J*). There was also clear expression of HLA class I in many cardiac myocytes (Fig. 1, *B, E,* and *H*) as well as fibroblasts (Fig. 1, *E, H,* and *K*) and ICAM-1 in many fibroblasts (Fig. 1, *C, F, I,* and *L*) and vascular endothelium (Fig. 1 *F*) in the myocardial tissue in all cases. We also examined the expression of perforin, HLA class I, and ICAM-1 in myocardial biopsy samples from two other patients with acute myocarditis and three other patients with dilated cardiomyopathy. We found again the expression of perforin in infiltrating cells and expression of HLA class I as well as ICAM-1 in myocardial cells in all these patients (data not shown). The expression of HLA class I and ICAM-1 in the myocardial cells might enable these cells to be the target cells for infiltrating killer lymphocytes.

Expression of TCR Vα and Vβ genes in PBLs of a normal subject. To examine the efficiency of the 5′-Vα and 5′-Vβ primers, we analyzed the expression of TCR Vα and Vβ genes in PBLs of a normal subject. Fig. 2 (*A* and *B,* PBL) shows the results of Southern blot analysis. Almost all TCR Vα as well as Vβ genes were expressed in PBLs of a normal subject. These results indicate that the PCR using the Vα and Cα as well as Vβ and Cβ primers listed in Table 1 could amplify the individual Vα and Vβ gene transcripts.

Restricted expression of TCR Vα and Vβ genes in infiltrating cells in the heart with acute myocarditis and dilated cardiomyopathy. To investigate TCR Vα and Vβ usage at the site of inflammation, Vα and Vβ transcripts in infiltrating cells in the hearts of patients with acute myocarditis and dilated cardiomyopathy were amplified by the PCR method. Fig. 2 shows the results of Southern blot analysis of the PCR-amplified products obtained from infiltrating cells in the hearts with acute myocarditis (Fig. 2, cases 1 and 2) and dilated cardiomyopathy (Fig. 2, cases 3 to 6). As shown in Fig. 2, in contrast to the expression in PBLs, only a few or limited number of Vα as well as Vβ genes were preferentially rearranged and transcribed in infiltrat-

Table III. Relative Distribution of Phenotypic Markers Among Infiltrating Cells in the Heart of Patients With Acute Myocarditis and Dilated Cardiomyopathy

Case No.	Age (yr)	Sex	Cells positive for (%)					
			CD4	CD8	CD14	CD16	CD20	TCR $\gamma\delta$
1	35	M	3.0	29.3	31.0	19.0	5.0	2.3
2	33	F	17.8	24.3	27.1	24.5	0.5	2.5
3	55	M	19.0	13.8	24.0	22.8	0.0	6.5
4	39	M	12.8	11.3	25.8	25.9	0.0	0.5
5	29	M	9.5	12.8	18.0	36.8	0.5	5.1
6	57	M	8.7	12.4	19.8	25.0	1.5	5.5
Mean±S.E.	41.33±4.41		11.80±2.24	17.32±2.82	24.28±1.79	25.67±2.23	1.25±0.71	3.73±0.86

400 cells were counted for each marker.

ing cells in the hearts of all patients. Although there were no specific patterns in the expression of Vα and Vβ genes among these patients, Vα16 and Vβ14 were rearranged in 3 of 6 patients, Vα2, Vβ3, Vβ4, and Vβ13.1 were rearranged in 2 of 6 patients.

Failure to detect enterovirus genomes in the myocardial tissues of patients with acute myocarditis and dilated cardiomyopathy. To investigate whether or not enterovirus genomes persistently existed in the cytoplasm of myocardial cells in these patients, we examined the expression of enterovirus genomes by PCR. Fig. 3 shows the results of Southern blot analysis of PCR-amplified enterovirus genomes. As shown in Fig. 3, PCR products from the myocardial tissue of C3H/He mice on day 7 of CVB3-infection (Fig. 3, C3H mouse [D7]) showed significant levels of enterovirus genomes, confirming that the primers used in this study could amplify enterovirus genomes effectively as previously reported (18). In contrast, there was no detectable levels of enterovirus genomes in the PCR products from myocardial tissues of all patients with acute myocarditis and dilated cardiomyopathy (Fig. 3, cases 1–6).

Discussion

In this study, we demonstrated that there were infiltration of perforin-expressing killer cells and enhanced expression of HLA class I and ICAM-1 in myocardial tissues of patients with dilated cardiomyopathy as well as acute myocarditis, providing the evidence that an autoimmune process may play a critical role in the pathophysiology of dilated cardiomyopathy. We also demonstrated that TCR Vα as well as Vβ gene usage by infiltrating cells in the hearts of these patients was restricted, indicating that the infiltrating cells specifically recognized and damaged the myocardial cells in the hearts with dilated cardiomyopathy.

Evidence has accumulated that there was active inflammation in the hearts of not a few patients with dilated cardiomyopathy. Furthermore, in some portion of them, even with a history of heart failure of several-years' duration, there were numerous lymphocytic clusters and myocardial cell destruction with extensive fibrosis in the hearts, presenting features of chronic myocarditis (22, 23). Our data in the present study confirmed that cell-mediated cytotoxicity really occurred in the hearts with dilated cardiomyopathy as those with acute myocarditis.

As for acute murine viral myocarditis, we previously reported that NK cells infiltrated the heart first and damaged the virus-infected cardiac myocytes, then T cells infiltrated secondly and might play a critical role in myocardial damage in the later stage (10). Considering the immunopathology involved in chronic myocarditis leading to dilated cardiomyopathy as the consequence of acute myocarditis, persistent expression of HLA and ICAM-1 on myocardial cells, as revealed in the present study, might enable them to be antigen-presenting cells for infiltrating T cells and cause persistent myocardial damage. If T cell–mediated autoimmune process plays a major role in the pathogenesis of dilated cardiomyopathy, the analysis of the T cell repertoire at the site of inflammation is of great importance. In particular, characterization of the TCR repertoire in the heart could be a direct way to examine the importance of T cells in the pathogenesis of dilated cardiomyopathy. Using an acute myocarditis model of C3H/He mice induced by CVB3, we have recently demonstrated that TCR Vβ gene usage by infiltrating T cells in the heart on day 12 after virus inoculation was restricted (16). We also found that TCR Vα gene usage in this model of acute myocarditis was restricted (unpublished data). This indicates that a specific antigen, presented at the groove of MHC molecules, in the heart was targeted. Because viral RNA persists in the heart at least up to 4 wk after virus inoculation (24), it is strongly suggested that the antigen recognized by the infiltrating T cells in acute murine myocarditis was virus-derived peptides. In a murine model of dilated cardiomyopathy, viral RNA could be detected by PCR in the chronic stage of encephalomyocarditis virus-induced myocarditis of DBA/2 mice, up to 90 d after virus inoculation when diffuse myocardial fibrosis was prominent, whereas myocardial necrosis and cell infiltration had disappeared (25). On the other hand, in the present study, although there were moderate cell infiltration and widespread myocardial necrosis with moderate interstitial fibrosis in the hearts with dilated cardiomyopathy, no enterovirus genomes were detected in the myocardial tissues of all patients, who were in the last stage of heart failure. Other studies on human samples from patients with myocarditis or dilated cardiomyopathy (18, 26–28) revealed that there was discrepancy between the presence of enteroviral genomes and histologic findings of inflammation. This indicates that the findings of viral persistence do not necessarily implicate the pathogenesis and strongly suggested that once viral infection triggers immunological responses, an autoimmune process may develop regardless of the viral persistence. There is also a possibility

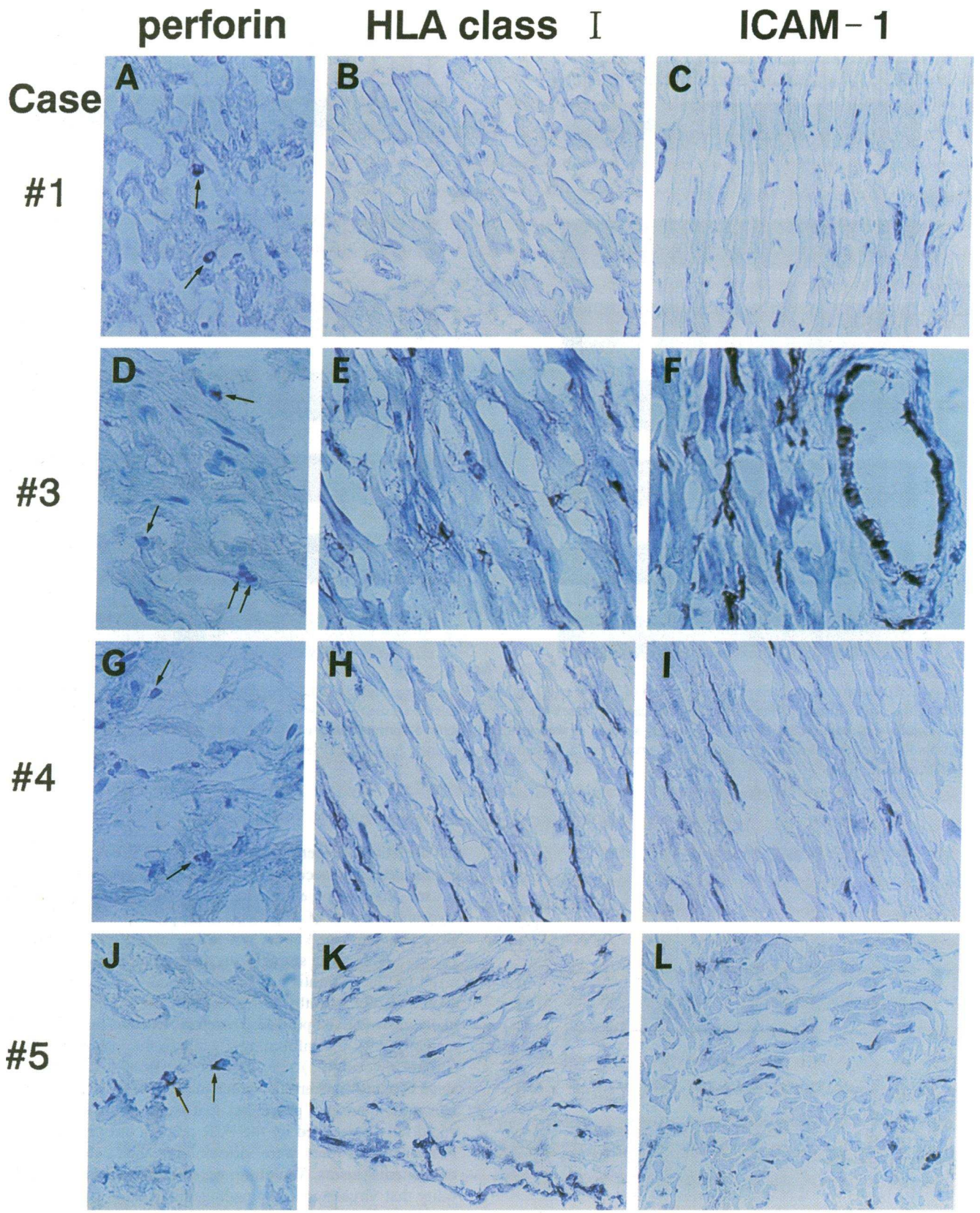

Figure 1. Immunohistochemical study of myocardial tissues from patients with acute myocarditis (case 1) and dilated cardiomyopathy (cases 3–5) for perforin (*A, D, G,* and *J*), HLA class I (*B, E, H,* and *K*), and ICAM-1 (*C, F, I,* and *L*). original magnification, ×200.

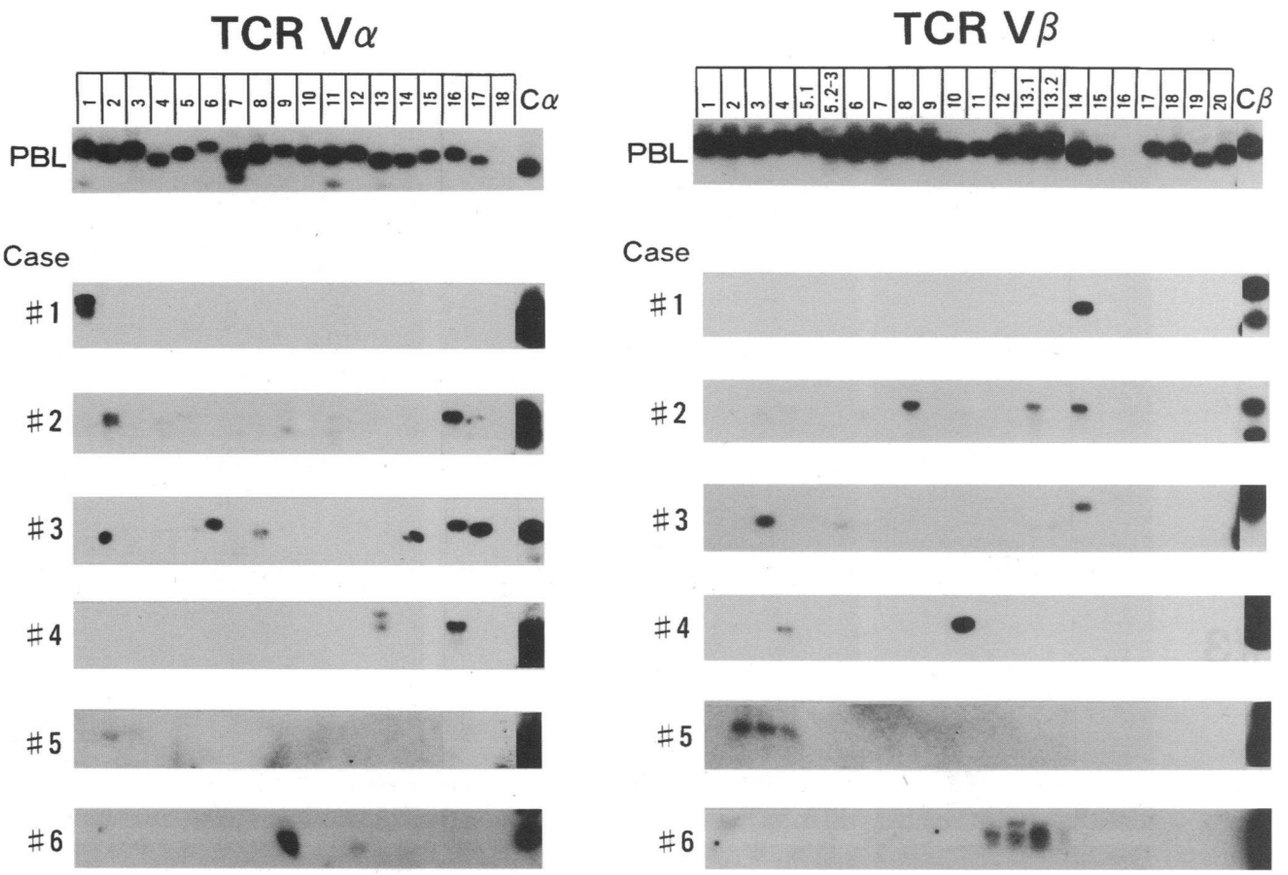

Figure 2. Southern blot analysis of the expression of TCR Vα (*A*) and Vβ (*B*) gene segments in PBLs from a normal subject (PBL), and infiltrating cells in the hearts from patients with acute myocarditis (cases 1 and 2) and dilated cardiomyopathy (cases 3–6). In all samples, the PCR was performed using each of the 18 TCR Vα family-specific as well as a Cα-specific 5′-primer and a common Cα 3′-primer, or each of the 22 TCR Vβ family-specific as well as a Cβ-specific 5′-primer and a common Cβ 3′-primer. Amplified DNAs were hybridized with a ^{32}P-labeled 5′-Cα or 5′-Cβ primer.

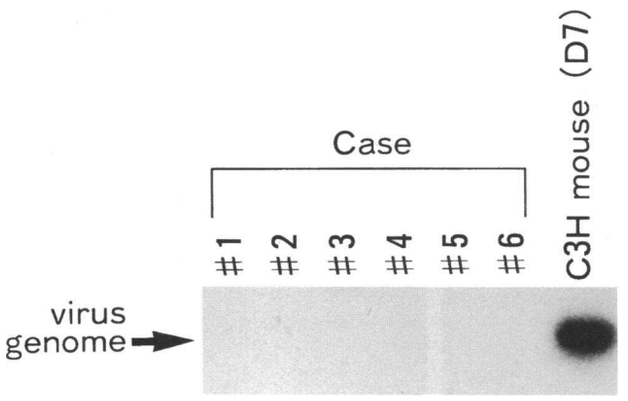

Figure 3. Southern blot analysis of the expression of enterovirus genomes in myocardial tissues from patients with acute myocarditis (cases 1 and 2) and dilated cardiomyopathy (cases 3–6) as well as from C3H/He mice on day 7 of CVB3-infection. In all samples, the PCR was performed using enterovirus-specific primers. Amplified DNAs were hybridized with a ^{32}P-labeled CVB3 cDNA probe.

that detection of enteroviral genomes by PCR or in situ hybridization does not necessarily mean the presence of infectious viruses. Because no enterovirus genomes were detected in the myocardial tissues of all patients in the present study and enterovirus genomes are known not to be integrated into the host genomic DNA, it is strongly suggested that the antigen recognized by the infiltrating T cells, especially in the hearts with dilated cardiomyopathy with a history of several years, was not likely virus-derived peptide. Therefore, it seems that the antigen recognized by the infiltrating T cells in the late stage (that is chronic myocarditis) may be different from that in the early stage of acute myocarditis, which may be virus-derived peptide. However, there is a possibility that some of the enteroviruses could not be detected by the PCR primers used in the present study and that there were viruses other than enteroviruses in the patients' hearts. Therefore, we could not exclude the possibility that viruses could be pathogenic in these patients.

We hypothesize that viral infection triggers the immunological responses which result in the enhanced expression of HLA and ICAM-1 on myocardial cells and that infiltrating T cells recognize virus-derived antigen and damage virus-infected myocardial cells in the early stage. Then, after virus genomes had disappeared, infiltrating T cells recognize a different anti-

gen, which may be a kind of autoantigen, presented by HLA along with ICAM-1 on myocardial cells and cause persistent myocardial damage in the late stage. Although we could not analyze the TCR gene usage in the early stage as well as in the late stage in the same patient, it seems that the pathogenic T cell clones in the early stage (which may be specific to virus-antigen) are different from those in the late stage (which may be specific to autoantigen), rather than that the same pathogenic T cell clones remain continuously present in the myocardium during the evolution from acute myocarditis to dilated cardiomyopathy. The reason why enhanced expression of HLA and ICAM-1 on myocardial cells continue after virus genomes had disappeared and what kind of antigen do infiltrating T cells recognize in the late stage are still unknown and remain to be clarified.

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