T Cells With Similar T-Cell Receptor β-Chain Complementarity-Determining Region 3 Motifs Infiltrate Inflammatory Lesions of Synthetic Peptides Inducing Rat Autoimmune Myocarditis

Haruo Hanawa, Takayuki Inomata, Yuji Okura, Satoru Hirono, Yusuke Ogawa, Tohru Izumi, Makoto Kodama, Yoshifusa Aizawa

Abstract—Experimental autoimmune myocarditis (EAM) resembles the giant cell myocarditis seen in humans, and recurrent forms lead to dilated cardiomyopathy. EAM has been shown to be a T cell–mediated autoimmune myocarditis. We have previously shown that cDNA encoding Vβ complementarity-determining region (CDR) 3 from heart– and pericardial space–infiltrating T cells in EAM induced by rod cardiac myosin contains more restricted sequences than that from normal spleen T cells. Recently, it has become apparent that several epitopes of EAM exist in rod cardiac myosin; therefore, T cells infiltrating into lesions may recognize certain epitopes in EAM induced by rod cardiac myosin. In this study, we examined heart– and pericardial space–infiltrating T-cell clonotypes in EAM induced by synthetic peptides of cardiac myosin. EAM was produced by immunization with synthetic peptides corresponding to N-terminally acetylated amino acids 1539 to 1555 of rat cardiac myosin α heavy chain. Five of 12 rats receiving synthetic peptides developed macroscopic signs of myocarditis. To examine T-cell receptor (TCR) Vβ expression and CDR3 of the TCR β chain of lesion-infiltrating T cells in EAM, total RNA was isolated from heart, pericardial effusion, spleen, lymph node, and peripheral blood. TCR Vβ expression of the T cells infiltrating the lesions revealed a predominance of Vβ4. On the basis of single-strand conformation polymorphism analysis for CDR3 of the TCR Vβ4 chain, heart– and pericardial space–infiltrating T cells were considered to be oligoclonal, whereas spleen, lymph node, and peripheral blood in a rat with EAM and spleen in a native rat were considered to be polyclonal. In the same rat, clonotypes of heart-infiltrating T cells were almost the same as those of pericardial space–infiltrating T cells. Furthermore, on sequence analysis for CDR3 of the TCR Vβ4 chain, the amino acid motifs were similar among T cells infiltrating into lesions of different EAM rats. In the present study, TCR β chains of heart– and pericardial space–infiltrating T cells in EAM induced by synthesized peptide consisting of 17 amino acids were examined. Vβ4+ T cells with similar Vβ CDR3 motifs that infiltrate the heart and pericardial space may recognize the same epitope. (Circ Res. 1998;83:133-140.)

Key Words: myocarditis ■ T-cell receptor ■ epitope ■ cardiomyopathy ■ immune system

EAM induced by injecting cardiac myosin into susceptible strains of rats and mice resembles the giant cell myocarditis seen in humans, and recurrent forms lead to dilated cardiomyopathy.1–3 EAM in rats is characterized by cardio-megaly, pericardial effusion, and extensive myocardial necrosis. Mononuclear cells, which are almost exclusively macrophages and CD4+ T cells, massively infiltrate the heart and pericardial space.4,5 EAM has been shown to be a T cell–mediated autoimmune disease by adoptive transfer, and the prevention of EAM by anti-αβ TCR antibody indicates the important role of αβ T cells.6–8

αβ T cells recognize antigenic peptides in the context of major histocompatibility as a result of interaction of the peptide major histocompatibility complex with the α and β chains.9 In the TCR α chain, CDR3 contacts the NH2-terminal portion of the peptide, whereas the TCR β chain contacts the COOH-terminal portion of the peptide.10

A previous study has shown oligoclonal expansion of T cells in lesions from rats with EAM induced by rod cardiac myosin.11 These oligoclonal T cells may recognize specific antigens, release cytokines to initiate myocarditis, and activate macrophages that release NO to injure myocytes.12,13 More recently, it has become apparent that there are several epitopes of EAM in rod cardiac myosin.14–18 We indicated that the myocardiogenic epitopes in Lewis rats were located in the RDCB9 (residues 1070 to 1165) of the rod cardiac myosin and that a cryptic minor epitope may reside in S-1.17 On the other hand, a study by Wegmann et al19 demonstrated that epitopes in Lewis rat EAM were located in the CM1 (residues 1304 to 1320) and CM2 (residues 1539 to 1555) of the
cardiac myosin. To identify CM peptides with myocarditogenic properties, Wegmann et al made use of a putative binding motif for the major histocompatibility class II molecule RT1.B1 in the rat. Because there are several epitopes of EAM in rod cardiac myosin, T cells in lesions from rats with EAM induced by rod cardiac myosin may recognize certain antigenic peptides. Such T cells may constitute groups that recognize different antigenic peptides. In the present study, we elucidate T-cell clonality in inflammatory lesions from rats with EAM induced by synthetic peptide CM2, which may be a single epitope.

Materials and Methods

Animals

Lewis rats were obtained from Charles River, Japan (Atsugi, Kanagawa, Japan) and were maintained in our animal facilities until they reached 6 weeks of age.

Induction of EAM

N-terminal acetylated synthetic peptides corresponding to aa 1539 to 1555 (CM2) of rat cardiac myosin α heavy chain were constructed and purified by Sawady Technology Co. CM2 peptides were dissolved in PBS at a concentration of 4 mg/mL and emulsified with an equal volume of complete Freund's adjuvant supplemented with 10 mg/mL of Mycobacterium tuberculosis H37RA (Difco). To produce actively induced EAM, on day 0, rats received a single immunization at 4 subcutaneous sites on the foot with a total of 0.4 mL of emulsion for each rat, thus yielding an immunizing dose of 800 μg of CM peptide per rat.

RT-PCR Analysis of Vβ Expression

Lymph nodes and spleen mononuclear cells were isolated by forcing popliteal lymph node and spleen through a 200-gauge stainless steel mesh. Red blood cells in the spleen cell preparation were lysed in 0.17 mol/L Tris supplemented with 0.83% NH4 Cl. Mononuclear cells in peripheral blood were isolated by Ficoll-Paque (Pharmacia) density gradient centrifugation at 1000 g for 20 minutes. Mononuclear cells in pericardial effusion were isolated after hemolysis in 0.17 mol/L Tris supplemented with 0.83% NH4Cl. Total RNA was isolated from heart, lymph node, spleen, peripheral blood, and pericardial effusion by acid guanidinium thiocyanate–phenol–chloroform extraction. cDNA was synthesized from 10 μg of total RNA with a TCR Cβ primer and murine Moloney leukemia virus reverse transcriptase (GIBCO-BRL) in a final volume of 20 μL. Vβ-specific PCR products were generated with AmpliTaq polymerase (Toyobo Co), 22 Vβ-specific primers, and the same Cβ primer from 1 μL of heart cDNA and 0.2 μL of lymph node, spleen, peripheral blood, and pericardial effusion DNA.

Figure 1. RT-PCR analysis of TCR Vβ gene expression in the spleen of a native Lewis rat (A), the heart (B) and pericardial effusion (C) of 3 EAM rats on day 17, and the heart of an EAM rat on day 20 (D). The ethidium bromide–stained agarose gels of the RT-PCR products are shown. No TCR signal was detected from identical analyses performed on native rat heart (data not shown). Lanes on the left contain ϕX174 digested with HaeIII.

Figure 2. Clonal analysis of TCR Vβ4 transcripts from the spleen of a native rat (A), the heart (B) and pericardial effusion (C) of an EAM rat on day 17, the heart of an EAM rat on day 20 (D), and the heart (E) and pericardial effusion (F) of an EAM rat on day 23. Silver-stained nondenaturing polyacrylamide gels of PCR products are shown. The arrows indicate bands in smear. No TCR signal was detected from identical analyses performed on native rat heart (data not shown).

Figure 3. Clonal analysis of TCR Vβ4 transcripts from the spleen (A) and lymph node (B) of an EAM rat on day 17, the spleen of another EAM rat on day 17 (C), and the peripheral blood of an EAM rat on day 23 (D). Top, Silver-stained nonde- naturing polyacrylamide gels of PCR products are shown. Bottom, The ethidium bromide–stained agarose gels of the RT-PCR products are shown. No TCR signal was detected from identical analyses performed on native rat heart (data not shown). Lanes on the left contain ϕX174 digested with HaeIII.

Selected Abbreviations and Acronyms

CDR = complementarity-determining region
CM peptide = cardiac myosin peptide
EAM = experimental autoimmune myocarditis
HLA = human lymphocyte antigen
PCR = polymerase chain reaction
RT = reverse transcription
TCR = T-cell receptor
pericardial effusion cDNA, according to the following amplification profile: 30 cycles at 94°C for 60 seconds, 55°C for 90 seconds, and 72°C for 120 seconds. Amplified products were separated on 3% agarose gels and stained with ethidium bromide.

**PCR Single-Strand Conformation Polymorphism**

Amplified PCR products were diluted (2:3) in denaturing solution (95% formamide, 10 mmol/L EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), heated at 96°C for 5 minutes, and then cooled on ice for 10 minutes. The diluted sample (10 μL) was adjusted to 50% glycerol, and 3 μL was subjected to single-strand conformation polymorphism by electrophoresis on nondenaturing 5% polyacrylamide (ratio of acrylamide to bisacrylamide, 19:1) gel (14 cm × 14 cm × 0.1 cm) in 0.5 Tris-borate-EDTA containing 10% glycerol at 14 V/cm for 5 hours at room temperature. DNA fragments were detected with a silver staining kit (Daiichi Chemical Co Ltd).

**Sequencing Analysis**

For the purposes of cloning and sequencing, Vβ-specific PCR products were purified with microcon-100 (Amicon Inc) and directly inserted into the pGEM-T vector (Promega). The recombinant plasmids were then used to transform *Escherichia coli* JM109 competent cells (Takara). Individual ampicillin-resistant colonies were isolated, and the plasmid DNA was sequenced with a TaqDye Deoxy terminator cycle sequencing kit and a DNA sequencer (model 373A or 310, Applied Biosystems).

**Results**

**Induction of EAM**

Twelve rats injected with CM2 peptide were killed on day 14 (3 rats), day 17 (5 rats), day 20 (2 rats), and day 23 (2 rats). The 3 rats killed on day 14 did not have macroscopic signs of myocarditis. However, 3 of the 5 rats (Nos. 1, 2, and 3) killed on day 17, 1 of the 2 rats killed on day 20, and 1 of the 2 rats killed on day 23 had developed small macroscopic signs of myocarditis. Four of the rats with myocarditis, except for the rat killed on day 20, had massive pericardial effusion.

**RT-PCR Analysis of TCR Vβ Expression**

We compared TCR Vβ expression in the heart and pericardial effusion of the 3 rats with myocarditis on day 17 (early stage) and the rat with myocarditis on day 20 with that of mononuclear cells from normal rat spleen, because a marked bias toward the expression of Vβ8.2 was apparent early during the onset of experimental autoimmune encephalomyelitis induced by guinea pig myelin basic protein in Lewis rats. RNA was extracted from spotty macroscopic white lesions in the hearts of EAM rats. PCR analysis revealed that the expression of Vβ4 genes in the hearts and pericardial spaces of EAM rats was more apparent than the expression in normal rat spleen (Figure 1).

**PCR Single-Strand Conformation Polymorphism Analysis of TCR Vβ4 Chain CDR3**

The structure of PCR products corresponding to the CDR3 region of the TCRβ chain was examined by single-strand conformation polymorphism analysis. Heterogeneous PCR products corresponding to this region migrate as a smear on single-strand conformation polymorphism analysis, whereas homogeneous products migrate as bands. Because Vβ4 genes were the dominant genes expressed in the hearts and pericardial spaces of EAM rats, we examined TCR Vβ4 chain CDR3. PCR products of TCR Vβ4 chain from normal spleen and from individual EAM heart and pericardial effusion on day 17, 20, or 23 migrated as a single band on ethidium bromide–stained agarose gels (Figure 1 and data not shown). However, on single-strand conformation polymorphism analysis, PCR products from normal spleen produced a smear, whereas those from EAM heart and pericardial effusion generated bands within a smear (Figure 2). This shows that oligoclonal T cells expanded in inflammatory lesions in the heart and pericardial effusion but not in normal spleen.

Furthermore, the fact that the PCR products from heart and pericardial effusion in the same EAM rat generated the same bands indicates that the clonality of T cells was the same in the heart and pericardial effusion.

We examined the TCR Vβ4 chain CDR3 region from popliteal lymph node, spleen, and peripheral blood of EAM rats by single-strand conformation polymorphism analysis (Figure 3). It was demonstrated that the cells were polyclonal, unlike the T cells from the heart and pericardial effusion. It demonstrated that oligoclonal T cells expanded only in

**Table 1. Vβ4 Sequences from Mononuclear Cells Isolated from the Spleen of a Native Lewis Rat**

<table>
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<tr>
<th>Vβ</th>
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<th>Jβ</th>
<th>No. of identical sequences</th>
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![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Vβ4 sequences from mononuclear cells isolated from the spleen of a native Lewis rat. Underlined nucleotides indicate variations from the reported Lewis Vβ and Jβ sequences and may be attributable to polymerase error.25
Figure 5. Vβ4 sequences from the hearts and pericardial effusions of 3 EAM Lewis rats on day 17. Vβ4 sequences from the heart (A) and pericardial effusion (B) of No. 1 EAM, from the heart (C) and pericardial effusion (D) of No. 2 EAM, and from the heart (E) and pericardial effusion (F) of No. 3 EAM. Letters indicate the cDNA clones that have identical sequences. Numbers on the right indicate cDNA clones that have similar amino acid CDR3 motifs. Seven amino acids of the Vβ side of CDR3 were compared with each of the cDNA clones, and cDNA clones differing in ≥2 amino acid motifs were regarded as having similar amino acid motifs. Seven amino acids surrounded by squares with the same mark are similar. Underlined nucleotides indicate variations from the reported Lewis Vβ and Jβ sequences and may be attributable to polymerase error. N indicates N region; D, D region.
inflammatory lesions, such as those found in the heart and pericardial effusion.

Sequence Analysis of TCR Vβ4 Chain CDR3

CDR3 sequences of Vβ4 cDNA from normal spleen revealed no oligoclonal expansion (Figure 4). In contrast, their sequences of cDNA from 3 EAM hearts or pericardial effusions on day 17 (Figure 5), an EAM heart on day 20 (Figure 6), and an EAM heart or pericardial effusion on day 23 (Figure 7) revealed oligoclonal expansion. In individual rats, their sequences of Vβ4 cDNA from the heart were the same as those from pericardial effusion. To sum up, single-strand conformation polymorphism and sequence analysis indicated that the same clones expanded in the heart and pericardial space. Although these analyses in popliteal lymph node, spleen, and peripheral blood of EAM rats showed polyclonality, some cDNA clones were the same as those of inflammatory lesions of identical EAM rats (Figure 8). On the other hand, CDR3 sequences of Vβ4 cDNA from the hearts and pericardial spaces of different EAM rats were similar. Seven amino acids of the Vβ side of CDR3 were compared with each of the cDNA clones, and cDNA clones differing in <2 amino acid motifs were detected (Figures 5, 6, and 7). No such cDNA clones were found in normal spleen (Figure 4).

Discussion

We examined heart– and pericardial space–infiltrating T-cell clonotypes in EAM induced by synthetic peptides of rat cardiac myosin. Vβ expression of T cells infiltrating into lesions revealed a predominance of Vβ4, and amino acid CDR3 motifs of the TCR Vβ4 chain were similar in different EAM rats. Previously, we determined the clonotypes of heart– and pericardial space–infiltrating αβT cells in EAM induced by porcine rod cardiac myosin, and we also noted that the cDNA encoding Vβ3 CDR3 from inflammatory lesions of EAM contained more restricted sequences than that from normal spleen. However, the expression of the TCR β chain V gene was not restricted at the site of inflammation, and T cells with similar amino acid motifs of CDR3 did not infiltrate into inflammatory lesions in different EAM rats. The synthetic peptide used as the myocarditogenic antigen in the present study consisted of 17 amino acids, and major histocompatibility class II molecules are considered to bind peptide antigen consisting of 15 to 18 amino acids; thus, this synthetic peptide is suspected to be a single myocarditogenic epitope. We consider that in EAM induced by the single myocarditogenic epitope, T cells do not respond to other epitopes in rod cardiac myosin; therefore, infiltrating T cells have restricted Vβ gene usage with similar amino acid motifs of CDR3.

It is interesting that the CDR3 sequences in hearts and pericardial spaces of individual EAM rats were the same; however, those of different EAM rats were not the same, but they were similar. We suppose that not one but several myocarditogenic T cells can respond to this synthetic peptide.
epitope. Therefore, in one EAM rat a few clones of myocarditogenic T cells may be activated, and in another EAM rat a few other clones of myocarditogenic T cells may be activated. T cell clones that were cultivated by the same antigenic peptide were reported to have different kinds of TCR with similar CDR3, 25 and in recent studies, it was reported that contact with TCR and antigenic peptide in the context major histocompatibility was not strict and that a kind

Figure 8. \(V_b\) sequences from spleen (A) and popliteal lymph node (B) of No. 2 EAM on day 17, from the spleen of No. 3 EAM on day 17 (C), and peripheral blood of EAM on day 20 (D). Letters, numbers, and squares are as for Figure 5. Underlined nucleotides indicate variations from the reported Lewis \(V_b\) sequences and may be attributable to polymerase error. 52

N indicates N region; D, D region.

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138 T-Cell Clonotypes in Synthetic Peptides Inducing EAM
of T-cell clone could respond to several antigenic peptides. 26
The present study is compatible with those reports.

Why pericardial effusion arises after immunization with a contractile protein such as myosin heavy chain or synthetic cardiac myosin is very interesting. In EAM, epicardial lesions are more severe than endocardial lesions. In a previous study, 4 we showed that many inflammatory cells might infiltrate the heart from the pericardial space. Myocarditogenic T cells that are autoreactive to myosin heavy chain or synthetic cardiac myosin may make contact with antigen-presenting cells in the epicardial region, and T-cell proliferation may occur in the pericardial cavity.

Experimental autoimmune encephalomyelitis, which is similar to multiple sclerosis, is induced in susceptible animal strains by immunization with myelin basic protein. In Lewis rats, the recognition of myelin basic protein clones 68 to 88 is carried out by TCR homologous to Vβ8.2, and myelin basic protein clones 87 to 99 are Vβ specific. 19,27 Restriction in TCR usage in pathogenic clones provided a rationale for therapies that prevented and reversed experimental autoimmune encephalomyelitis with Vβ-specific monoclonal antibodies. 29 The CDR3 of TCRβ chains expressed by these clones shows similarities in rats with experimental autoimmune encephalomyelitis. 24,26 Remarkably, T cells with the amino acid motif LRG in the CDR3 region are found in lesions produced by experimental autoimmune encephalomyelitis in the Lewis rat after immunization with myelin basic proteins 87 to 99 and in lesions of patients with multiple sclerosis who exhibit HLA DR2. 19,30 T cell clones in Lewis rats and in patients with multiple sclerosis, which express this CDR3 motif, all have been reported to have specificity for myelin basic protein 87 to 99. 19,30 If this is so, it is conceivable that a common therapy for multiple sclerosis and animal experimental autoimmune encephalomyelitis can be established.

Myocarditis and dilated cardiomyopathy in humans and in animal models has been studied intensively. Several viruses, 30–34 especially coxsackievirus, are thought to cause human myocarditis or dilated cardiomyopathy, whereas genetic regulation in the host and the interplay between virus persistence and autoimmunity are speculated to produce the later phases of the disease. 35 In the pathogenesis of myocarditis and dilated cardiomyopathy, cellular immune responses are considered to play an important role, 36,37 and T cells within inflammatory lesions were reported to use a restricted TCR V region 38–41 or TCR CDR3 region 11; however, the mechanism of heart-specific T-cell responses is not yet clear.

B- and T-cell epitopes of myocarditis or dilated cardiomyopathy have been reported by other investigators. 40–46 Recently, T-cell epitopes of mouse and rat T-cell–mediated EAM were reported. 45,48 If the epitope of animal EAM is also the epitope of human myocarditis or dilated cardiomyopathy (e.g., myelin basic protein 87 to 99 of experimental autoimmune encephalomyelitis and multiple sclerosis), then the CDR3 of the TCR expressed by T-cell clones should show similarities in EAM and human myocarditis or dilated cardiomyopathy, and it is conceivable that a common therapy for human myocarditis or dilated cardiomyopathy and EAM can be established.

HLA class II (DR and DQ) antigens are suspected to be associated with dilated cardiomyopathy, 47,48 and studies of HLA in dilated cardiomyopathy are now being undertaken. Recent immunological studies have characterized the motifs for peptide binding to major histocompatibility classes I and II. 39,49 To elucidate the mechanism of myocarditis or dilated cardiomyopathy and establish specific immunotherapies, further studies are needed to clarify the relation between major histocompatibility of patients, pathogenic epitopes, and TCR of pathogenic T cells.

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