C Protein-Induced Myocarditis and Subsequent Dilated Cardiomyopathy: Rescue from Death and Prevention of Dilated Cardiomyopathy by Chemokine Receptor DNA Therapy

Yoh Matsumoto, Yukiko Tsukada, Akira Miyakoshi, Hiroshi Sakuma and Kuniko Kohyama

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C Protein-Induced Myocarditis and Subsequent Dilated Cardiomyopathy: Rescue from Death and Prevention of Dilated Cardiomyopathy by Chemokine Receptor DNA Therapy

Yoh Matsumoto, Yukiko Tsukada, Akira Miyakoshi, Hiroshi Sakuma, and Kuniko Kohyama

Severe experimental autoimmune myocarditis and subsequent dilated cardiomyopathy (DCM) were successfully produced in Lewis rats by immunization with recombinant cardiac C protein. Seventy-five percent of immunized rats died between days 15 and 49 postimmunization, and all of the survived rats showed typical DCM characterized by the presence of ventricular dilatation and extensive fibrosis. Immunopathological and chemokine analysis during the acute phase revealed that there were marked macrophage infiltration with myocyte necrosis and up-regulation of MCP-1 and IFN-γ-inducible protein-10 (IP-10). Based on these findings, we prepared plasmid DNAs encoding the binding site of CCR2 and CXCR3, which are receptors for MCP-1 and IP-10, respectively. The culture supernatant of cells transfected with these DNAs inhibited the migration of T cells and macrophages induced by MCP-1 and IP-10. Remarkably, administration of the DNAs to C protein-immunized rats prevented the disease progression and rescued animals from death. The present study has demonstrated for the first time that gene therapy targeting the chemokine receptor could be a powerful tool for the control of experimental autoimmune myocarditis and DCM. The Journal of Immunology, 2004, 173: 3535–3541.

Dilated cardiomyopathy (DCM) is a serious disorder and the most common cause of heart failure. Although a certain proportion of DCM may be genetic in origin, the majorities are sporadic, and a viral or immune mechanism is suspected (1). The incidence of biopsy-proved myocarditis varies greatly among series of patients with DCM, ranging from 1 to 67% (2), but examinations of large tissues obtained by partial ventriculectomy (Batista operation) revealed that more or less inflammatory lesions were found in all of the cases (3). In addition, it was reported that cardiac inflammation was found in some apparently healthy relatives of patients with DCM, suggesting that inflammatory processes, either autoimmune or nonautoimmune, may be involved in the development of DCM (4). Based on these findings, several immunosuppressive therapies including steroid and azathioprine administration (5, 6), immunoabsorption (7), and Ig therapy (1) were tried to improve the status of DCM. To date, significant progress has not been made, although these therapies showed some improvements of the disease. Difficulties in finding effective therapies are mainly based on the fact that the pathogenesis of DCM is still poorly understood. The establishment of an animal model that mimics human DCM will provide useful information with regard to the pathogenesis of DCM and the development of effective therapies.

Experimental autoimmune myocarditis (EAC) is an animal model for human myocarditis and DCM, and has served for the elucidation of the pathomechanism of the disease and development of immunotherapy. Cardiac myosin has been considered as a major cardiitis-inducing Ag. Curiously, however, highly purified myosin and synthetic peptides corresponding to various parts of the myosin molecule reduce their carditogenicity drastically (8–11). This finding raises the possibility that cardiac proteins other than myosin play an essential role in the development of EAC. In addition, we recently found that skeletal C protein induced more severe experimental autoimmune polymyositis than purified skeletal myosin (12). These findings strongly suggest that cardiac C protein rather than myosin is a major cardiitis-inducing Ag. However, it is difficult to obtain highly purified native C protein at a sufficient amount for the induction of EAC.

In the present study, we prepared recombinant C protein to overcome the above problems and succeeded in inducing severe EAC in Lewis rats and subsequent DCM in survivors by immunization with the protein. The pathological features of the heart at the chronic phase of the disease fulfill the criteria for the presence of DCM (the presence of extensive fibrosis, marked ventricular dilatation, and increase in the heart weight compared with the normal heart) (11). Using this animal model, we demonstrate in this study that chemokines such as MCP-1 and IFN-γ-inducible protein-10 (IP-10) are highly up-regulated during the acute phase, and that neutralization therapy with chemokine receptor DNAs targeting these chemokines can block the progression of the disease and rescue animals from death due to cardiac failure. Analysis of C protein-induced EAC provides not only insights into pathogenesis of autoimmune myocarditis and subsequent DCM, but also useful information regarding development of effective immunotherapies against the disease.
Materials and Methods

Animals and proteins

Lewis rats were purchased from SLC Japan (Shizuoka City, Japan) and bred in our animal facility. Rats used in the present study were 6–12 wk old. Partially purified and purified cardiac myosin and C protein were prepared, as described previously (12).

Preparation of recombinant C protein fragments

Because C protein is too large to prepare recombinant protein as a whole protein, we planned to produce four protein fragments designated as fragments 1, 2, 3, and 4. Total RNA was isolated from human heart using RNAzol B (Biotex Laboratories, Houston, TX) and then reverse transcribed into cDNA using ReverTra Ace-PolyA (Toyobo). The cloned cDNA was PCR amplified with KOD DNA polymerase (Toyobo) and fragment-specific primer pairs. Primers used in this study are listed in Table I. Each primer was designed to possess the restriction enzyme site at both ends. PCR products were inserted into a cloning vector, pCR4-Blunt-TOPO (Invitrogen Life Technologies, CH Groningen, The Netherlands), and clones with right sequences were obtained by the manufacturer’s instructions. Colonies grown in competent cells were picked, and recombinant plasmid DNA was isolated using Mini prep (Promega). Then the constructs were inserted into the vector containing human Ig Fc portion. By restriction enzyme digestion, colonies with an insert with right direction and length were screened, and the nucleotide sequence of each clone was determined to confirm that inserts had the right sequence with ATG in frame. Large-scale preparation of plasmid DNA was performed using Qiagen EndoFree Plasmid Mega Kit (Qiagen). DNAs at a total dose of 100 μg in 100 μl of 0.25% bupivacaine (Sigma-Aldrich, St. Louis, MO) were injected into bilateral tibialis anterior muscles using the indicated protocols.

Migration inhibitory assay

HEK293 cells were seeded onto 35-mm dishes (Corning, Tokyo, Japan) at a density of 2 × 10^5 cells/well in 2 ml of DMEM (Sigma-Aldrich) supplemented with 10% FCS and kanamycin and cultured overnight. Plasmid DNAs encoding rat CCR2-Fc or CXCR3-Fc genes were transfected into the cells using DMRIE-C reagent (Invitrogen Life Technologies). The supernatants were collected and stored at −80°C. The chemotaxis assay was conducted using a CHEMOTAX chemotaxis chamber with a 5-μm pore size (NeuroProbe, Gaithersburg, MD), as described previously (15–17). Splenic T cells were suspended at 10^6 cells/ml in Gey’s balanced salt solution containing 2% BSA and applied to the upper wells of the chamber (25 μl/well). IP-10 at 10 or 50 ng/ml was preincubated with or without culture supernatant containing CXCR3 for 1 h at 37°C and applied to lower wells (30 μl/well). After 2 h at 37°C, T cells that had migrated into the lower wells were counted. All assays were done in triplicate. In the MCP-1 chemotaxis assay, glycogen-stimulated peritoneal macrophages were applied to the upper well and MCP-1 preincubated with or without culture supernatant containing CCR2 was placed in the lower well.

Gene therapy with decoy chemokine receptor-encoding DNA

Rats were immunized with recombinant C protein fragment 2 and received i.m. administration of chemokine receptor DNAs (100 μg), alone or in combination, from days 1 to 28 postimmunization three times per week. The effect of treatment was evaluated at the indicated time points by histological examinations.

Production of chemokine receptor fusion proteins from plasmid DNAs and ELISA

pTARGET plasmid encoding the biding site of CXCR3 gene fused with human Ig Fc gene was digested with restriction enzymes and subcloned into pQE30 (Qiagen). The sequence of the construct was confirmed by sequencing. rCXCR3 produced in transformed E. coli were isolated under

Table I. The primer pairs used in this study

<table>
<thead>
<tr>
<th>C Protein</th>
<th>Primer</th>
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<tbody>
<tr>
<td>Fragment 1</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGAGG-3’</td>
</tr>
<tr>
<td>Fragment 2</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGGAGG-3’</td>
</tr>
<tr>
<td>Fragment 3</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGGAGG-3’</td>
</tr>
<tr>
<td>Fragment 4</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGGAGG-3’</td>
</tr>
<tr>
<td>CCR2</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGGAGG-3’</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Sense = 5’-GAGGAGGATCATGCTGCTGAGGCGAGGAGGAGGAGGAGGAGG-3’</td>
</tr>
</tbody>
</table>
denaturing conditions and purified using Ni-NTA agarose (Qiagen). Then purified CXCR3 was diluted, refolded as described above.

The level of anti-CXCR3 Abs was measured by ELISA. rCXCR3 was coated onto microtiter plates, and serially diluted sera from treated animals were applied. After washing, appropriately diluted horseradish-conjugated anti-rat IgG was applied. The reaction products were then visualized after incubation with the substrate. The absorbance was read at 450 nm.

Results

Autoimmune carditis-inducing ability of partially purified myosin, purified myosin, and native C protein isolated from human heart tissue

To characterize the Ag(s) responsible for the development of EAC in more detail, we further purified cardiac myosin by using a DEAE Sepharose column and immunized it in Lewis rats. The results are summarized in Table II. Although partially purified myosin induced severe EAC with a mean histological score of 2.8 ± 0.2, as expected, purified cardiac myosin induced significantly milder EAC (score 1.9 ± 0.2) (group A vs B, p < 0.05). In addition, there was a significant difference in heart weight between the two groups (p < 0.01). These findings raised the possibility that protein(s) other than myosin in the partially purified cardiac myosin preparation is responsible for the development of EAC. One of the candidate Ags is C protein, a 150-kDa myosin-binding protein, which was reported to induce EAC (18) and experimental autoimmune polymyositis (12). As suspected, the partially purified myosin preparation contained a large amount of 150-kDa protein, as revealed by SDS-PAGE electrophoresis (data not shown). Based on these findings, we examined the myocarditis-inducing ability of C protein using purified native protein. The results are shown in Table II, group C. Unexpectedly, native C protein elicited only mild EAC in three of eight rats. This was probably because the purity and immunogenicity of native C protein obtained in this experiment were low (data not shown). To overcome these problems and to confirm the carditis-inducing ability of C protein, we decided to produce recombinant C protein, which would enable us to use a sufficient amount of highly purified protein.

Estimation of EAC-inducing ability of recombinant C protein fragments

Because cardiac C protein is too large to produce a single plasmid encoding the whole sequence of the protein, we prepared four plasmids encoding four parts of cardiac C protein. Fragments 1, 2, 3, and 4 correspond to the 1–323, 317–647, 641–970, and 964–1274 aa sequences, respectively. Then purified recombinant protein fragments in CFA were immunized in Lewis rats along with i.p. injection of PT, and hearts were taken at indicated time points for histological examination. The results are shown in Table II, groups D–G. Immunization with fragments 1, 2, 3, and 4 induced EAC in almost all of the rats. Fragments 1 and 2 induced severe EAC with marked cardiac hypertrophy (~3-fold heavier than normal hearts) and inflammation with a histological score of 3.3 (groups D and E). Immunization with fragments 3 and 4 resulted in the induction of slightly mild EAC (groups F and G). It should be noted, however, that all the four fragments possess a carditogenic activity, strongly suggesting that cardiac C protein contains multiple EAC-inducing epitopes over the entire molecule. Because immunization with skeletal C protein did not induce EAC, severe inflammation in the heart was shown to be cardiac C protein specific (data not shown). As fragment 2 is most hydrophilic and showed strong carditogenicity, subsequent studies were performed using this recombinant protein fragment.

Longitudinal study of EAC induced by immunization with recombinant C protein fragment 2

We immunized rats (n = 12) with cardiac C protein fragment 2 and followed the clinical course of EAC. As shown in Fig. 1A, 75% of immunized rats died between days 15 and 49 postimmunization, and all the survived rats showed typical DCM characterized by the presence of ventricular dilatation and extensive fibrosis (see below). Then hearts were taken from immunized rats at various time points and weighed, as shown in Fig. 1B. The hearts of rats 2–4 wk after immunization were 3- to 4-fold heavier than those of normal rats. The heart weight reached a maximal level at 4 wk and declined gradually until 12 wk.

Frozen and paraffin-embedded sections were stained with H&E for general pathology, as well as Azan for the connective tissue and immunohistochemistry for T cells and macrophages (Fig. 2). Fig. 2, A–D, was taken at the same magnification. The heart taken

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Table II. Histological severities of EAC induced by immunization with partially purified and purified cardiac myosin and native and recombinant C protein

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunogen</th>
<th>Sampling</th>
<th>Incidence of EAC</th>
<th>Heart Weight (g)</th>
<th>Histological Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Partially purified myosin</td>
<td>4 wk</td>
<td>4/4</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>B</td>
<td>Purified myosin</td>
<td>4 wk</td>
<td>4/4</td>
<td>0.8 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>C</td>
<td>Native C protein</td>
<td>4 wk</td>
<td>3/8</td>
<td>0.7 ± 0.1</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>D</td>
<td>C protein fragment 1 (1–323)</td>
<td>2 wk</td>
<td>3/3</td>
<td>1.5 ± 0.3</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>E</td>
<td>C protein fragment 2 (317–647)</td>
<td>2 wk</td>
<td>3/3</td>
<td>1.7 ± 0.2</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>F</td>
<td>C protein fragment 3 (641–970)</td>
<td>2 wk</td>
<td>3/3</td>
<td>1.4 ± 0.4</td>
<td>2.2 ± 1.2</td>
</tr>
<tr>
<td>G</td>
<td>C protein fragment 4 (964–1274)</td>
<td>2 wk</td>
<td>2/3</td>
<td>1.0 ± 0.2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* Lewis rats were immunized with partially purified myosin (1.5 mg), purified myosin (1.5 mg), or native or recombinant human myosin fragments (100 μg) in CFA in the footpads along with i.p. injection of PT (2 μg).

Significantly different between groups A and B (p < 0.05).

Significantly different between groups A and B (p < 0.01).

FIGURE 1. A, Survival rate of rats immunized with recombinant C protein fragment 2/CFA plus i.p. injection of PT. Seventy-five percent of the rats died between days 15 and 49. B, The heart weight of rats immunized with C protein fragment 2. The heart weight reached a maximal level at 2–4 wk and declined gradually until 12 wk.
fields counted = 6 for each staining). The average number of T cells was 64.0 ± 10.0, whereas that of macrophages was 172.2 ± 21.9 ($p = 0.001$). During the chronic phase (6 wk and thereafter), connective tissue expansion was marked around the vessels and between muscle fibers (Fig. 2, G and H).

**Cytokine and chemokine analysis of the hearts with EAC**

Based on the findings obtained by immunopathological examinations, we determined the levels of chemokines and proinflammatory cytokines at the RNA level by competitive PCR analysis. As shown in Fig. 3, MCP-1 increased gradually, reached a maximal level at 4 and 6 wk, and declined sharply 8 wk after immunization (Fig. 3A). IP-10 and TNF-α (Fig. 3, B and C) showed a similar pattern as MCP-1, but the up-regulation of IFN-γ was transient and restricted at 4 wk (Fig. 3D). We also confirmed using randomly selected samples that the kinetics of these chemokines and cytokines at the protein level paralleled well with the RNA level (data not shown).

**DNA therapy targeting chemokines involved in the development of EAC prevents the disease progression to DCM and rescues animals from death**

Immunopathological examination and chemokine analysis strongly suggest that MCP-1 and IP-10, chemoattractants for T cells and macrophages, play an essential role in the formation and maintenance of EAC lesions. To confirm their role and to establish immunotherapy targeting chemokines to control autoimmune processes, we prepared nonviral plasmid vectors inserted with DNAs encoding the binding sites of CCR2 and CXCR3, which are receptors for MCP-1 and IP-10, respectively. We first tested whether these DNAs really neutralize the function of MCP-1 and IP-10 in the in vitro migration assay. As shown in Fig. 4, culture supernatants from the CXCR3 DNA-transfected cells inhibited T cell migration induced by IP-10. Essentially the same results were obtained using CCR2 DNA (data not shown). Then rats were immunized with cardiac C protein fragment 2 and chemokine receptor DNAs, alone or in combination, injected three times per week from days 1 to 28 postimmunization, and sacrificed for histological evaluation at 6 wk. For control, an empty vector was

![FIGURE 2. Macroscopic findings of the heart taken from a normal rat (A), rats during the peak of the acute phase of EAC (B), and at the chronic phase (C and D). A–D were taken under the same magnification. During the peak of the acute phase, the heart shows marked expansion, and its lumen is almost obliterated (B). At the chronic phase, the diameter of the heart is almost the same as that at the acute phase (C). The most remarkable finding at this stage is prominent dilatation of the ventricle and extensive fibrosis, as demonstrated by Azan staining (D). Immunohistochemical examinations during the acute phase revealed severe infiltration of T cells (E) and macrophages (F). Note that the number of infiltrating macrophages is much larger than that of T cells. Pathology of the heart at the chronic phases (G and H) demonstrates extensive muscle fiber loss and intermyocardial fibrosis. A–C, H&E staining; D, Azan staining; E, R73 staining; F, ED1 staining; G, H&E staining; F, Azan staining.](http://www.jimmunol.org/content/197/8/3538/F2)

![FIGURE 3. Competitive PCR analysis of cytokines and chemokines in the heart of rats at various time points of EAC. mRNAs isolated from heart tissues were subject to competitive PCR analysis for MCP-1 (A), IP-10 (B), TNF-α (C), and IFN-γ (D). Three to five rats were examined at each time point.](http://www.jimmunol.org/content/197/8/3538/F3)
injected according to the same schedule. The results are summarized in Table III. In the control group, four of nine rats (44%) died during the observation period (group 4), and the heart weight of survived rats was 1.6 ± 0.2 g. Microscopic examination revealed that there was extensive fibrosis with a fibrosis score of 4.4. In sharp contrast, CXCR3 DNA-treated rats (group 1) did not die during the observation period, and the heart weight was markedly light (mean heart weight of 1.1 g); in addition, the mean fibrosis score was significantly lower than that of controls. Treatment with CCR2 and CXCR3 plus CCR2 DNAs also showed the significant protective effects against the development of DCM (groups 2 and 3). Pathological examinations demonstrated that in sharp contrast to untreated hearts (Fig. 5C), the size, weight, and extent of fibrosis of the hearts of treated rats (Fig. 5B) were almost the same as those of normal rats (Fig. 5A). Microscopically, virtually no inflammatory cell was found in the parenchyma (data not shown). We also measured the level of IP-10 mRNA in the hearts of rats treated with chemokine receptor DNAs or the empty vector (Fig. 5D). As clearly shown, IP-10 mRNA in the heart of rats treated with

![FIGURE 4. In vitro effects of CXCR3 DNA on IP-10-driven T cell migration. Cultured HEK293 cells were transfected with plasmid DNA encoding CXCR3, and the supernatants were harvested and incubated with IP-10. Then the chemotaxis assay was conducted using a chemotaxis chamber. Splenic T cells were applied to the upper well, and IP-10 preincubated with (f) or without (L) the culture supernatant containing CXCR3 was placed in the lower well. After 2 h at 37°C, cells that had migrated into the lower wells were counted.](image)

![FIGURE 5. Treatment of EAC and DCM with chemokine receptor DNA. A and B, Azan staining of hearts taken from normal (A), CXCR3 DNA-treated (B), and empty vector-administered (C) rats. All the photographs were taken under the same magnification. D, Competitive PCR quantitation of IP-10 mRNA in hearts of rats with or without chemokine receptor DNAs. DNAs (100 μg), alone or in combination, were administered i.m. from days 1 to 28 three times per week.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>DNA</th>
<th>Mortality (%)</th>
<th>Sampling</th>
<th>Heart Weight (g)</th>
<th>Fibrosis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CXCR3</td>
<td>0/6 (0%)</td>
<td>6 wk</td>
<td>1.1 ± 0.6c</td>
<td>1.4 ± 1.1d</td>
</tr>
<tr>
<td>2</td>
<td>CCR2</td>
<td>0/3 (0%)</td>
<td>6 wk</td>
<td>0.9 ± 1.2c</td>
<td>1.7 ± 1.2d</td>
</tr>
<tr>
<td>3</td>
<td>CXCR3 + CCR2</td>
<td>0/3 (0%)</td>
<td>6 wk</td>
<td>0.7 ± 0.1c</td>
<td>1.5 ± 0.8d</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>0/9 (44%)</td>
<td>6 wk</td>
<td>1.6 ± 0.2c</td>
<td>4.4 ± 0.4d</td>
</tr>
</tbody>
</table>

Table III. Treatment with plasmid DNAs encoding chemokine receptor DNA rescues C protein-immunized animals from death and prevents the development of DCM

* Lewis rats were immunized with C protein fragment 2 (100 μg) in CFA in the footpads with PT, and the indicated DNAs (100 μg) were injected every other day from day 1 postimmunization until day 28.

The extent of fibrosis revealed by Azan staining was graded into five categories: grade 1, rare scattered foci of fibrosis; grade 2, multiple isolated foci of fibrosis; grade 3, fibrosis involving outer layer of the muscle; grade 4, grade 3 plus partial transmural fibrosis; grade 5, diffuse fibrosis.

Significantly different between groups A and D (p = 0.0003), between groups B and D (p = 0.003), and between groups C and D (p = 0.0004) by Student’s t test or Welch’s test.

Significantly different between groups A and D (p = 0.0007), between groups B and D (p = 0.007), and between groups C and D (p = 0.0006) by Student’s t test or Welch’s test.
CXCR3 DNA was significantly suppressed compared with that in the control group. Interestingly, the level of IP-10 mRNA in CCR2 DNA- and CXCR2 plus CCR2 DNA-treated rats was also low. These findings clearly indicate that DNA therapy targeting chemokines ceased the progression of EAC and subsequent DCM and rescued animals from death.

It was important to determine whether chemokine receptor DNA therapy induces autoantibodies against the chemokine protein produced by administered plasmid DNA. To examine this issue, we injected CXCR3 DNA three times per week for 4 wk. Sera taken at the end of the treatment were subjected to ELISA using rCXCR3. As clearly shown in Fig. 6, there was no significant Ab elevation in treated animals.

Discussion

EAC is an animal model for human autoimmune and virus-induced myocarditis and inflammatory cardiomyopathy (19). In the majority of studies, cardiac myosin was used as a myocarditis-inducing Ag. In the process of identifying carditogenic epitopes using synthetic peptides, it was found that residues 334–352, 406–425, 406–425, 947–960, 1539–1555, and 1631–1650 in the cardiac myosin H chain molecule were carditogenic in mice (10, 11, 20) and rats (8). However, these highly purified myosin preparations markedly reduced their carditogenicity compared with native partially purified protein, suggesting that there are other carditogenic proteins in this preparation. Based on the findings obtained in a previous report (18) and our experience in the polymyositis model (12), we decided to examine the carditogenicity of cardiac C protein. Consequently, we succeeded in producing severe EAC and subsequent DCM in Lewis rats by immunization with recombinant cardiac C protein, a myosin-binding protein (21), and also found that C protein possesses multiple highly carditogenic epitopes in the entire molecule. Because C protein is located on the surface of the myosin molecule (21, 22) and is hydrophilic compared with self aggregatable myosin, it is likely that C protein is more carditogenic than myosin. A similar phenomenon is observed in the CNS, where myelin oligodendrocyte glycoprotein, which is located on the outer surface of the myelin sheath, is more encephalitogenic than myelin basic protein located inside the myelin component, and induces long-lasting encephalomyelitis (23).

By the use of recombinant C protein, we were able to induce severe EAC and subsequent DCM in Lewis rats. Immunohistochemical examination revealed that there was marked infiltration of macrophages into the myocardium during the acute phase. Consistent with this finding, chemokines that induce macrophage migration were highly up-regulated in the heart. These findings strongly suggest that infiltrating macrophages rather than T cells are the final effector cells in the disease process. Although previous studies showed that EAC was transferable into naive animals by injection of sensitized T cells (24, 25), we have not succeeded to produce passive EAC in this experimental system by adoptive transfer of C protein- or Con A-stimulated T cells from C protein-immunized rats (our unpublished observation). These findings suggest that not only T cells, but also macrophages and humoral factors are involved in the lesion formation during the acute phase. The processes from autoimmune inflammation to DCM with extensive fibrosis remain poorly understood. Several factors such as the renin-angiotensin system (26), edema (27), and complements (28) produced by either inflammatory or noninflammatory mechanism may be involved in this disease process. Very recently, two interesting reports were published. Okazaki et al. (29) demonstrated that autoantibodies against cardiac troponin I are responsible for DCM in programmed cell death-1-deficient mice, and that administration of anti-cardiac troponin I mAb induced DCM in wild-type mice. Eriksson et al. (30) reported that mild autoinflammation in the heart and subsequent activation of innate immunity result in the development of DCM. In this study, we demonstrated for the first time that autoimmunity against cardiac C protein and chemokine secretion in the heart play a major role in the development of DCM.

Suppression or blockade of pathways involved in the disease process can be effective therapies for DCM. Immunosuppressive treatment (6) and angiotensin-converting enzyme inhibitor (31) showed some effects in preventing the disease progression in humans and animals. In this study, we demonstrated that supernatants from cells transfected with DNAs encoding the binding sites of CC2 and CXCR3 suppress the function of their ligands, MCP-1 and IP-10, respectively, in vitro, and that administration of these DNAs to C protein-immunized rats stops the progression of EAC and subsequent DCM, rescuing animals from death. The mechanism by which in vivo administration of chemokine receptor DNAs suppresses the progression of EAC and DCM remains to be elucidated. At present, we postulate that decay chemokine receptors derived from injected DNAs enter the heart tissue and function as suppressive agents by binding chemokines in the target organ, thus inhibiting the development of inflammatory lesions. This is the reason that not only CXCR3, but also CCR2 DNA treatment down-regulated the level of IP-10 in the heart. It is also generally accepted that chemokines function during the primary immune responses, which take place in the regional lymph node (32). Our speculation does not exclude the possibility that chemokine receptor DNAs exert their suppressive effects in the lymphoid organ. Of interest was that the treatment protocol used in this study did not induce autoantibodies against chemokine receptors, as revealed by ELISA (Fig. 6). As the uptake of plasmid DNA by muscle cells after i.m. injection was estimated to be <1% of injected dose (33), the levels of translated proteins would be too low to elicit the Ab production. This assumption is also supported by the finding that immunohistochemical examination of translated proteins (the binding site of chemokine receptor fused with human Ig Fc portion) using anti-Fc Abs failed to detect positive signals in both the lymphoid organ and heart (data not shown).

In summary, severe EAC and subsequent DCM in survivors were successfully produced in Lewis rats by immunization with recombinant cardiac C protein. Administration of DNAs encoding CCR2 and CXCR3, which are receptors for MCP-1 and IP-10, to
immunized rats prevented disease progression and rescued animals from death. These findings strongly suggest that treatment of autoimmune inflammation in the heart, whether it is subtle or obvious, is essential to prevent the development of DCM. Analysis of C protein-induced EAC provides not only insights into the pathogenesis of human autoimmune myocarditis and DCM, but also useful information regarding the development of effective immunotherapies against the disease.

Acknowledgment
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References