In Vivo Lymphocyte-Mediated Myocardial Injuries Demonstrated by Adoptive Transfer of Experimental Autoimmune Myocarditis

Makoto Kodama, MD; Yoh Matsumoto, MD; and Michio Fujiwara, MD

Background. To elucidate the mechanisms of immune-related myocardial injuries, we examined whether autoimmune myocarditis was passively transferable by use of humoral or cellular factors.

Methods and Results. Active myocarditis was elicited in Lewis rats by immunization with human cardiac myosin fraction in complete Freund’s adjuvant. This experimental myocarditis was characterized by macroscopic features such as pericardial effusion, enlargement of the heart, and gray discoloration of the cardiac surface. Histologically, extensive myocardial necrosis and numerous inflammatory cell infiltrations were observed. Interestingly, multinucleated giant cells were frequently observed in the lesions. Transfer of the disease by the humoral factor was examined by use of fresh sera and immunoglobulin fraction of pooled sera from rats with severe myocarditis, and transfer by the cellular factor was tested by use of spleen cells and lymph node cells from the diseased rats. When naive Lewis rats were given 15.75 mg of immunoglobulin fraction, no particular change was observed in the hearts. Fresh sera also could not elicit myocarditis in recipient rats. In contrast, intravenous injection of spleen cells or lymph node cells that were cultured for 3 days in the presence of 1 μg/ml of concanavalin A elicited severe myocarditis. The macroscopic and microscopic findings of passively transferred myocarditis are essentially the same as those found in actively induced myocarditis. Multinucleated giant cells were also observed in the lesions of transferred myocarditis.

Conclusions. This study demonstrates direct evidence for in vivo lymphocyte-mediated myocardial injuries. (Circulation 1992;85:1918–1926)

Key Words • myocarditis • cells, T • cells, giant

Immunological mechanisms are responsible for the pathogenesis of several heart diseases, such as rheumatic carditis, chronic Chagas’ disease, Dressler syndrome, and postpericardiotomy syndrome,1–3 as well as myocarditis and dilated cardiomyopathy.4–9 Several reports indicate the etiologic relevance of anti-heart autoantibodies in dilated cardiomyopathy.10 In other reports, the significance of T cells was also demonstrated in experimental myocarditis.11 In clinical studies, however, direct demonstration of in vivo effects of humoral or cellular factors for myocardial injuries is difficult. Therefore, it is important to prepare an appropriate model for elucidation of the mechanisms of immune-related myocardial injuries.

We have recently established a novel experimental model of autoimmune myocarditis in rats by immunization with cardiac myosin that was characterized by extremely severe myocardial lesions and multinucleated giant cells.12 Various forms of experimental autoimmune myocarditis have been reported previously.13–20 However, the in vivo effects of humoral or cellular factors for myocardial injuries remain unclear. If the disease has an actual autoimmune nature, the disease can be transferred into syngeneic animals with antibodies or T cells,21–25

In this study, we investigated whether autoantibodies or lymphocytes from rats immunized with cardiac myosin could transfer the disease into syngeneic rats. We sought direct in vivo evidence for humoral or cellular mechanisms in this model of organ-specific autoimmune disease.

Methods

Animals

Inbred Lewis rats were purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan) and maintained at the Facilities for Comparative Medicine and Animal Experimentation, Niigata University School of Medicine.

Antigen

Cardiac myosin was prepared from the ventricular muscle of human hearts according to the method of Murakami et al26 with some modifications.12 The purification procedure contained several extraction and precipitation steps. All procedures were carried out at 4°C. Sample hearts were obtained at autopsy from...
patients who died of malignancy and had no history of myocarditis or heart failure. The purity of this antigen was checked by sodium dodecyl-polyacrylamide gel electrophoresis. The antigen preparation was composed chiefly of 205-kd protein but contained, at least in small amounts, several substances other than cardiac myosin.

Active Induction of Myocarditis

Cardiac myosin fraction was dissolved at a concentration of 10 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl. The antigen solution was mixed with an equal volume of complete Freund’s adjuvant supplemented with Mycobacterium tuberculosis H37Ra (CFA suppl H37Ra) (Difco, Detroit, Mich.) at a concentration of 11 mg/ml. On days 0 and 7, 7-week-old rats were injected into their foot pads with 1.0 mg s.c. of antigen in CFA suppl H37Ra (myosin group). On days 1 and 3, the rats were injected with 1.0 ml i.v. Bordetella pertussis vaccine (2×10^9/ml) (Nacalai Tesque, Kyoto). Age- and sex-matched Lewis rats served as controls. They were injected with PBS in CFA suppl H37Ra followed by injection with B. pertussis (PBS group). As another control for antigen, rats were immunized with 1.0 mg bovine serum albumin (BSA) in CFA suppl H37Ra followed by injection with B. pertussis (BSA group). All rats were killed under ether anesthesia on day 21.

Humoral Transfer

Serum was collected from rats with severe myocarditis (myosin group) and was stored at −80°C until use. Five syngeneic rats were injected with 5 ml i.p. nontreated sera (fresh sera group). They were killed on day 14. In the next experiment, saturated ammonium sulfate solution adjusted to pH 7.0 was added to the serum, and the fraction under 33% saturation was collected. After dialysis against PBS, the immunoglobulin fraction was reconstituted with PBS. Protein concentration of this solution was 3.5 mg/ml. Naive syngeneic rats were injected with 4.5 ml i.p. of this solution (IgG fraction group). Antibodies against cardiac myosin were measured by ELISA, and this solution was positive until dilution of 1,600:1. Passive myocarditis was assessed on days 11, 14, 21, and 28.

Cellular Transfer With Freshly Prepared Cells

Spleens were removed from rats with overt myocarditis, and single-cell suspension was prepared by passing through a stainless steel mesh screen. A single-cell suspension was washed three times in RPMI-1640 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% nonessential amino acids (GIBCO Laboratories), 0.4% (vol/vol) of penicillin-streptomycin mixture (Bioproducts, Inc., Md.), and 5×10^−5 M 2-mercaptoethanol and immediately injected intravenously into syngeneic rats at a dose of 1×10^7 viable cells per rat (fresh spl. group). The rats were killed on days 14 and 28 for histological examination.

Transfer With Activated Cells

Spleen cells and lymph node cells from the rats with autoimmune myocarditis were suspended at a density of 2×10^8 cells/ml in RPMI-1640 supplemented with the above-mentioned substances and cultured for 3 days in the presence of 1 μg/ml of concanavalin A (Con A) (Sigma Chemical, St. Louis). Cultured spleen cells or lymph node cells at doses of 1×10^7, 3×10^7, 1×10^8, and 3×10^9 viable cells were injected intravenously into syngeneic rats (Con A spl. and Con A LN groups). Recipient rats were killed on day 14 for histological examination.

Time Course

The next experiments were designed to clarify the onset and time course of adoptively transferred myocarditis. Lewis rats were injected with 3×10^7 lymph node cells that were cultured for 3 days in RPMI-1640 supplemented with the above-mentioned materials in the presence of Con A. The rats were killed on days 8, 11, 14, 21, and 28.

Mitogen

The effects of different mitogens on activation of myocarditogenic lymphocytes were investigated with phytohemagglutinin (PHA) and lipopolysaccharide (LPS). A single-cell suspension of lymph node cells was cultured in RPMI-1640 supplemented with the above-mentioned substances and containing, instead of Con A, 1/1,600% (vol/vol) of PHA (Difco) or 0.025 mg/ml of LPS (Sigma). Syngeneic rats were injected intravenously with 3×10^7 of these respective viable cells (PHA LN and LPS LN groups, respectively). Histological examination was carried out on day 14. Some part of the single-cell suspension of spleen cells was cultured in mitogen-free medium. Cultured cells at a dose of 3×10^7 were injected intravenously into syngeneic rats (cultured spl. group). Spleen cells from the rats of the BSA group were cultured by the same procedure and transferred at a dose of 2×10^7 viable cells per rat (BSA-primed Con A spl. group). Recipient rats were killed on day 21 for histological examination.

Histopathology

At the time of death, the presence of pericardial effusion and macroscopic findings of the hearts were investigated. Macroscopic findings were graded into three categories: 0, normal; 1, presence of focal discolored area; and 2, presence of multiple or diffuse discolored areas on the cardiac surface. Hearts were removed immediately after death and fixed in 10% formalin. After they were embedded in paraffin, transverse sections were cut at several levels of the hearts. The basal part of the heart was sliced longitudinally. These sections were stained with hematoxylin and eosin. Microscopic findings were graded as follows: 0, normal; 1, presence of a few small lesions, not exceeding 0.25 mm^2 in size, in a single section; 2, presence of multiple small lesions or a few moderate-sized lesions, not exceeding 6.25 mm^2, that correspond to the transmural lesions of the right ventricular free wall of the rats; and 3, presence of multiple moderate-sized lesions or larger lesions that correspond to the transmural lesions of the left ventricle of the rats.

Statistical Analysis

Results were expressed as mean±SD. Student’s t test was used for comparison of data. Differences were considered significant at p<0.05.
TABLE 1. Induction of Experimental Autoimmune Myocarditis

<table>
<thead>
<tr>
<th>Group</th>
<th>Diseased/total (%)</th>
<th>PE</th>
<th>Macroscopic scores (mean)</th>
<th>Microscopic scores (mean)</th>
<th>Body wt (initial) (g)</th>
<th>Body wt (last) (g)</th>
<th>Heart wt (g)</th>
<th>Heart wt/body wt (g/kg)</th>
<th>Deaths (%)</th>
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<td>3.0</td>
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<td>166±15*</td>
<td>1.42±0.44*</td>
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<td>0</td>
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<td>182±15§</td>
<td>0.72±0.02</td>
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PE, pericardial effusion.
*Two rats that died on days 19 and 20 were excluded here.
†Three rats died on days 19, 20, and 21.
‡p=NS between immunized group and control group.
§p<0.05.
‖p<0.001.

Results

Histopathology of Experimental Autoimmune Myocarditis

All the rats immunized with cardiac myosin had ruffled fur and were crouching down all day by the third week. Three of 16 rats in the myosin group died before they were due to be killed (Table 1). The hearts of 15 of 16 rats in the myosin group had massive pericardial effusion and were markedly enlarged (Figure 1). The index of heart weight/body weight was significantly higher in the myosin group than in the PBS group. There were gray discolored areas on the cardiac surface of all the rats of the myosin group. The discolored areas did not correspond to coronary perfusion areas (Figure 2A). The centers of the lesions were composed of various kinds of inflammatory cells, such as fragments of necrotic myocardial fibers, mononuclear cells, polymorphonuclear neutrophils, and eosinophils (Figure 3A). In the peripheries of the inflammatory lesions, mainly mononuclear cells were observed. One of the interesting findings was the appearance of multinucleated giant cells in the inflammatory lesions (Figure 3B). The giant cells had 10–20 nuclei that were arranged in a circular or horseshoe shape or gathered in the center of cytoplasm. Inflammatory lesions also appeared in the atria, appendixes, papillary muscles, and paracardiac ganglions but not in the valve leaflets, the wall of great vessels, or skeletal muscle. Rats of the PBS group showed no myocardial lesions. Rats of both the myosin group and the PBS group had small inflammatory lesions in their lungs and liver, which were probably systemic involvement of the adjuvant.

Humoral Transfer

Five rats injected with fresh nontreated sera from the rats with severe myocarditis were killed on day 14...
Rats that were injected with immunoglobulin fraction were killed for histological examination on days 11, 14, 21, and 28. None of the rats from those groups had pericardial effusion, and the surface color of the hearts was normal (Figure 4). No particular changes were detected at histological examination (Figure 2B).

**Cellular Transfer**

None of the rats injected with fresh spleen cells from sensitized rats at a dose of \( 1 \times 10^6 \) cells showed pathological changes in the heart on day 14 (Table 2). Otherwise, only one rat of five injected with fresh spleen cells developed mild myocarditis on day 28. In contrast, severe myocarditis characterized by macroscopic changes was elicited in the rats injected with Con A–activated spleen cells (Figure 4). As in actively induced myocarditis, the cardiac lesions of rats with transferred myocarditis were composed of myocardial necrosis and mixed cellular infiltration, predominantly lymphocytes and macrophages (Figure 5A). Multinucleated giant cells also appeared in the lesions of transferred myocarditis (Figure 5B).

**Sufficient Doses of Cells for Adoptive Transfer**

The dose of activated lymphocytes sufficient for adoptive transfer of autoimmune myocarditis was investigated (Table 3). None of the rats injected with Con A–activated spleen cells at doses of \( 1 \times 10^6 \) and \( 3 \times 10^6 \) developed myocarditis. Four of five rats injected with \( 1 \times 10^7 \) activated spleen cells showed myocarditis, some quite severe (Figure 2C). Some of the rats injected with \( 3 \times 10^7 \) lymph node cells developed myocarditis. Myocardial lesions observed in the rats injected with \( 3 \times 10^7 \) activated cells were as severe as those observed in the rats immunized with cardiac myosin, as shown in macroscopic and microscopic scores.

**Time Course of Transferred Myocarditis**

Rats injected with \( 3 \times 10^7 \) activated cells showed no myocardial lesions until day 8 after the cell transfer (Table 4). Transferred myocarditis was observed from day 11 after the injection. Pericardial effusion was observed in most of the rats killed on days 11 and 14. However, the rats killed on days 21 and 28 showed no pericardial effusion. The number of inflammatory cells decreased and fibrosis became prominent in myocardial lesions of the rats killed on day 21. Those findings were more apparent in the rats killed on day 28.

**Effects of Mitogens**

The effects of mitogen-free medium and various mitogens other than Con A were examined (Table 5).
FIGURE 3. Panel A: Histological section of lesion of experimental autoimmune myocarditis. Extensive myocardial necrosis was observed in the heart of immunized rats. The lesion was composed of fragments of necrotic fibers, neutrophils, and mononuclear cells. Bar indicates 200 μm. Panel B: Multinucleated giant cells appeared frequently in this myocarditis. Bar indicates 80 μm.

The rats injected with cells that were cultured in the mitogen-free medium for 3 days showed no myocardial lesions on day 21. No pathological findings were detected in the hearts of either group injected with PHA-activated cells and LPS-activated cells. No pathological findings were detected at death in the hearts of the rats immunized with BSA. The rats injected with Con A-activated spleen cells from the rats of the BSA group did not show myocarditis.

Discussion

Animal models are very useful for the analysis of autoimmune diseases. Regarding myocarditis, various experimental models have been reported that use the immunization procedure. In most of those models, however, only mild lesions appeared in the heart. In human acute myocarditis, cardiac enlargement and discoloration of the cardiac surface are common findings at autopsy. Pericardial effusion and congestive heart failure are frequently observed during the clinical course of human myocarditis. Only two reports, the guinea pig model by Hosenpud et al.28 and the murine model by Neu et al.29 showed enlargement of the heart with autoimmune myocarditis. However, discoloration of the cardiac surface, pericardial effusion, and lethal clinical course have not previously been reported in experimental autoimmune myocarditis. We were able to produce such pathological and clinical conditions by using the animal model reported here.

Giant-cell myocarditis is a lethal inflammatory heart disease.30–34 The cause of giant-cell myocarditis has not been clarified. Recently, circulating anti-heart antibod-

<table>
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<th>Group</th>
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<th>Day of death</th>
<th>PE</th>
<th>Macroscopic scores (mean)</th>
<th>Microscopic scores (mean)</th>
<th>Diseased/total</th>
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<td>IgG fraction</td>
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<td></td>
<td></td>
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<td>14</td>
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<td>0</td>
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<td>0/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>21</td>
<td>0/4</td>
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<td>0/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>28</td>
<td>0/4</td>
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<td>0</td>
<td>0/4</td>
</tr>
<tr>
<td>Fresh spl.</td>
<td>1×10⁶</td>
<td>5</td>
<td>14</td>
<td>0/5</td>
<td>0</td>
<td>0</td>
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<td>Fresh spl.</td>
<td>1×10⁷</td>
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<td>28</td>
<td>0/5</td>
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<td>0.2</td>
<td>1/5</td>
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<tr>
<td>Con A spl.</td>
<td>1×10⁴</td>
<td>5</td>
<td>14</td>
<td>2/5</td>
<td>1.0</td>
<td>2.2</td>
<td>4/5</td>
</tr>
</tbody>
</table>

PE, pericardial effusion; spl., spleen cells; Con A, concanavalin A.
ies were detected in the serum of a patient with this type of myocarditis.\textsuperscript{35} Occasionally, giant-cell myocarditis is associated with various immunological disorders, such as myasthenia gravis, ulcerative colitis, and pernicious anemia.\textsuperscript{36–38} Therefore, an autoimmune mechanism seems to be operating as a part of the pathogenesis, but...
no direct evidence for this could previously be obtained. For the first time, we were able to produce giant-cell myocarditis in Lewis rats using this immunization procedure. This model supports the possibility of autoimmune involvement in the pathogenesis of giant-cell myocarditis.

The significance of humoral factors in myocarditis has been reported by several investigators in clinical and experimental studies. In 1982, Maisch et al. reported the diagnostic and etiologic significance of anti-heart antibodies (anti-myolemmal antibodies) in patients with acute myocarditis. By in vitro assay, they demonstrated complement-dependent cytotoxicity of anti-myolemmal antibodies against heterologous cardiomyocytes. It has remained unclear, however, whether or not autoantibodies alone are able to elicit myocarditis in vivo.

Many investigations have supported the importance of cellular immunity in myocarditis, especially in Coxsackie virus B3 myocarditis. However, direct evidence for cell-mediated myocarditis has not been established in vivo. We have shown here, for the first time, in vivo evidence of lymphocyte-mediated myocardial injuries by adoptive transfer of sensitized lymphocytes. Severe myocarditis was transferred into naive Lewis rats by injection of cultured spleen cells or lymph node cells from previously immunized syngeneic rats. Because lymphocytes were activated by Con A in our culture system, the presence of Con A–reactive cells, i.e., T cells, seems to be necessary for the induction of myocarditis.

In this study, Con A–reactive spleen cells and Con A–reactive lymph node cells could similarly transfer myocarditis into syngeneic rats. However, freshly prepared spleen cells could not sufficiently transfer the disease. Because the lymphocytes were washed several times before transfer in all experimental groups, it is unlikely that the antigen or adjuvant used for the immunization, any pathogen, or substances contained in the culture medium played a role in the development of myocarditis in recipient rats. Therefore, we consider that 1×10^6 fresh spleen cells did not contain a high enough dose of activated effector T cells to elicit myocarditis. A similar phenomenon was reported in the system of experimental allergic encephalomyelitis.

We investigated the effects of three kinds of mitogens in activation of effector lymphocytes. The major action of each mitogen is considered to be directed to restricted subpopulations of lymphocytes; namely, Con A and PHA can stimulate T cells, and LPS can stimulate B cells. Because LPS was not effective in activation of effector cells, this myocarditis seemed to be a T cell-mediated autoimmune disease. The reason why PHA cannot sufficiently activate effector T cells is unknown, but a similar phenomenon can be observed in the experimental allergic encephalomyelitis system.

We have previously reported that the onset of actively induced experimental autoimmune myocarditis is about 16 days after the first immunization. From the present study, the onset of adoptively transferred myocarditis is about day 11 or earlier. Although the precise pathogenesis of this model has not yet been elucidated, in actively induced myocarditis, clonal expansion of effector T cells may be the first step, then recruitment of effector T cells to the target organ may follow. The last step is probably the effector–target interaction process. Because the initial clonal expansion of effector T cells is not necessary, the onset of transferred myocarditis occurs earlier. Clonal analysis of myocarditogenic lymphocytes is nec-

### Table 3. Sufficient Doses of Lymphocytes for Adoptive Transfer

<table>
<thead>
<tr>
<th>Group</th>
<th>Dosage</th>
<th>n</th>
<th>Day of death</th>
<th>PE</th>
<th>Macrosopic scores (mean)</th>
<th>Microscopic scores (mean)</th>
<th>Diseased/total</th>
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<td></td>
<td>3×10^6</td>
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<td>0/3</td>
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<td></td>
<td>1×10^7</td>
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PE, pericardial effusion; Con A, concanavalin A; spl., spleen cells; LN, lymph node cells.

*This group is the same as the group demonstrated in Table 2 (line 5).

### Table 4. Time Course of Adoptively Transferred Myocarditis

<table>
<thead>
<tr>
<th>Group</th>
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<th>Microscopic scores (mean)</th>
<th>Diseased/total</th>
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PE, pericardial effusion; Con A, concanavalin A; LN, lymph node cells.

*This group is the same as the group demonstrated in Table 3 (line 8).
nism of myocarditis.47,48

Our model of T cell–mediated, autoimmune myocarditis simulates closely the severity and histopathology of human giant-cell myocarditis. The action of viral infection, toxins, myocardial injury, or other etiologic agents may be similar to experimental immunization with cardiac myosin to induce autoimmunity to myocytes. Thus, this simple model should help to explore the pathogenesis of giant-cell myocarditis and perhaps other immune-mediated cardiomyopathies.

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<th>Group</th>
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<td>2 x 10^7</td>
<td>3</td>
<td>21</td>
<td>0/3</td>
<td>0</td>
<td>0</td>
<td>0/3</td>
</tr>
</tbody>
</table>

PE, pericardial effusion; Con A, concanavalin A; spl., spleen cells; LN, lymph node cells; PHA, phytohemagglutinin; LPS, lipopolysaccharide; BSA, bovine serum albumin.

†This group is also demonstrated in Table 2 (line 5) and in Table 3 (line 3).

*This group is also demonstrated in Table 3 (line 7).
43. Lawson CM, O’Donoghue H, Reed WD: The role of T cells in mouse cytoomegalovirus myocarditis. Immunology 1989;67:132–134
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