Prevention of Autoimmune Myocarditis Through the Induction of Antigen-Specific Peripheral Immune Tolerance

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Background—Autoimmunity to cardiac antigens, in particular cardiac myosin, has been observed in humans with myocarditis and in animals with experimental inflammatory heart disease. Current treatments for myocarditis are in many cases immunosuppressive and might lead to increased cardiac damage by reducing host defenses against infectious agents. Therefore, we sought to develop an antigen-specific approach to inhibit autoimmunity in mice with myosin-induced experimental autoimmune myocarditis.

Methods and Results—Syngeneic splenocytes, coupled with cardiac myosin by use of ethylene carbodiimide, were administered intravenously before disease induction, and the effects of this peripheral tolerization on myosin-induced myocarditis were assessed. This antigen-specific immunotherapy significantly reduced both the incidence and severity of myocarditis, with the prevention of myocyte necrosis, mononuclear cell infiltration, and fibrosis. Myosin-specific delayed-type hypersensitivity and antibody production were significantly reduced, demonstrating that peripheral tolerance affected both T- and B-cell responsiveness to the autoantigen.

Conclusions—These results suggest that the induction of antigen-specific peripheral immune tolerance may be an effective approach for the treatment of myocarditides with autoimmune involvement. (Circulation. 2001;103:1709-1714.)

Key Words: myocarditis  ■  myosin  ■  lymphocytes

Myocarditis is a primary inflammatory heart disease that is formally diagnosed by cardiac biopsy and observation of myocyte necrosis and degeneration, with adjacent mononuclear cell infiltration in the presence or absence of fibrosis. The most common causes of myocarditis in the United States are viral, most commonly coxsackievirus. Other infectious and noninfectious agents, such as bacteria, protozoans, fungi, drugs, heavy metals, and insect stings may also cause cardiac inflammation.

Immunomodulatory therapies, particularly immunosuppressive treatments, have been used in clinical trials for the treatment of myocarditis. Although immunosuppressive treatments are often useful in downregulating the autoimmune damage in myocarditis, they may promote viral spread and myocardial cytolysis. In fact, clinical trials and experimental studies of murine models of viral myocarditis have revealed that steroids and other immunosuppressive treatments are not always beneficial and may actually increase disease severity and mortality. Cytokine inhibition with vesnarinone has been shown to downregulate proinflammatory cytokine production, which would be deleterious during acute viral myocarditis. Conversely, such treatments as interleukin-2 and interferon-α may be effective in clearing the viral agent but may promote the development of cardiac autoimmunity.

Experimental autoimmune myocarditis (EAM) has proved to be a highly useful animal model of inflammatory heart disease. Myocarditis is induced on immunization of susceptible strains of rats and mice with the cardiac myosin α-heavy chain. Interestingly, immune responses against cardiac antigens, cardiac myosin in particular, have been observed in human inflammatory heart disease, making myosin a relevant antigen for disease induction in the mouse model. EAM is histologically similar to human myocarditis, with myocyte swelling and necrosis accompanied by mononuclear cell infiltration and fibrosis. EAM is a T cell–mediated disease, requiring both CD4+ and CD8+ subsets, whereas B cells are not vital for antigen presentation in EAM and autoantibodies are not necessary for the progression of myocarditis.

We wish to develop treatments to be used immediately on the suspicion of myocarditis without the necessity of determining the presence or absence of an infectious agent. Antigen-specific immunosuppression would allow for the inhibition of autoimmune damage without adversely affecting the beneficial immune response mounted against the infectious agent that initiated the damage. We and others have successfully used intravenous administration of syngeneic splenocytes covalently coupled to antigen with ethylene carbodiimide (ECDI) to prevent and, more importantly, to
treat a number of autoimmune diseases in animal models, such as experimental autoimmune encephalomyelitis, neuritis, uveitis, and thyroiditis, and initial application of this approach to humans has been successful. In this report, we demonstrate that coupled-cell tolerance is effective for prevention of myocarditis in this mouse model, suggesting the usefulness of antigen-specific immunotherapy in treating myocarditis.

Methods

Experimental Animals

Male A/J strain mice (n=142; Jackson Laboratories, Bar Harbor, Me) were 6 to 8 weeks of age at initiation of the experiments. Mice were anesthetized by a single injection of 60 mg/kg sodium pentobarbital IP for each experimental manipulation. The use and care of mice were in accordance with the guidelines of the Center for Experimental Animal Research at Northwestern University.

Preparation of Myosin

Cardiac myosin heavy chains were purified according to the method of Shiverick et al. Protein concentration was determined by comparing dilutions of the purified myosin solution with known concentrations of purified rabbit myosin heavy chain standards (Sigma) by SDS-PAGE.

Preparation of Myosin-Coupled Splenocytes

Splenocytes were purified from naive mice and incubated at 37°C for 10 minutes in Tris-NaCl (0.017 mol/L Tris free base, 0.14 mol/L NaCl, pH 7.2) to lyse erythrocytes, followed by 2 washes in HBSS (Gibco/BRL). The splenocytes were then coupled with purified cardiac myosin with the cross-linking chemical ECDI (Calbiochem) in the following manner. The splenocytes were washed in saline (0.15 mol/L NaCl, pH 7.2), pelleted at 1500g for 10 minutes in a 50-mL conical tube, and resuspended at a final concentration of 5×10^6 cells/mL in cold saline containing 1 mg/mL purified cardiac myosin or 1 mg/mL BSA (Sigma). The coupling reaction was initiated by the addition of 0.5 mL of freshly prepared ECDI (150 mg/mL in saline) per mL of the cell suspension and incubated for 1 hour on ice with periodic gentle inversion. The cells were washed 3 times with HBSS and maintained at 4°C until injection into mice.

Induction of Peripheral Tolerance

Tolerance was induced by the intravenous injection of 7.5×10^7 coupled splenocytes in 0.5 mL HBSS. Seven days after injection, the mice then received the normal myosin immunization protocol described below.

Induction of Autoimmune Myocarditis

Mice were immunized subcutaneously in 3 sites on the dorsal flank in a 100-μL volume containing 300 μg of purified cardiac myosin diluted 1:1 in complete Freund’s adjuvant (Difco). Seven days later, the mice were boosted with another 300 μg myosin in the same manner.

Histological Evaluation of Disease

Hearts were excised, fixed in 10% buffered formalin, and embedded in paraffin. Hearts were cut in a plane such that both atria and ventricles were present in each section. Four or more sections per heart were stained with hematoxylin and eosin or Masson’s trichrome and examined by microscopy. Evaluation of the heart sections was performed blinded. Disease severity was graded as follows: normal (0), no infiltrate; mild (1), <10% of the tissue section involved; moderate (2), 10% to 25% of the tissue section involved; and severe (3), >25% of the tissue section involved.

Measurement of Antigen-Specific Delayed-Type Hypersensitivity

Delayed-type hypersensitivity (DTH) responses were quantified with an ear-swelling assay 21 days after immunization. Mice were anesthetized, and prechallenge ear thickness was measured with a Mitutoyo model 7326 engineer’s micrometer (Mitutuyo MTI Corp). Antigen (10 μg) in a 10-μL volume of DTH buffer (0.15 mol/L K2HPO4, 0.01 mol/L Na2HPO4, 0.3 mol/L KCl, pH 6.8) was then injected intradermally into the dorsal surface of the ear with a 100-μL Hamilton syringe fitted with a 30-gauge needle. Ear swelling, resulting from mononuclear cell infiltration, was then measured 24 hours later and expressed in units of 10^-4 in.

Seroalogical Analysis

A myosin-specific ELISA was developed to analyze the isotype specificities of myosin-specific antibodies. After each incubation, the plates were washed 5 times with 1× PBST (1× PBS, 0.05% Tween-20). Ninety-six–well plates were coated with 2.5 μg/mL purified myosin overnight at room temperature and then blocked for 2 hours with 5% normal goat serum, 2% BSA in PBST. Mouse sera were diluted at 1:1000 in the blocking agent and incubated on plates for 2 hours at room temperature. The biotin-conjugated, isotype-specific secondary antibodies (Caltag Laboratories) were added at a dilution of 1:15 000 and allowed to adsorb for 1 hour. Peroxidase-labeled streptavidin (KPL Laboratories) was incubated at a dilution of 1:4000 for 30 minutes, and the plates were developed with TMB developing solution (KPL Laboratories) according to the manufacturer’s instructions. Each serum was analyzed separately, and the data were compiled for each group. Each plate contained the same myosin-positive (from a severely diseased EAM mouse) and -negative sera for normalization.

Statistical Analyses

All values are expressed as mean±SEM. The statistical significance of DTH, disease severity, and antibody isotypes was analyzed by 1-way ANOVA followed by post hoc Bonferroni analysis. The statistical significance of disease incidence was analyzed with Pearson’s χ². Probability values of P<0.05 were considered significant in this study.

Results

Induction of Myosin-Specific Tolerance in EAM

A/J mice, when immunized with syngeneic cardiac myosin, develop severe myocarditis 21 days after immunization. In the present study, this immunization protocol was coupled with a tolerogenic protocol in an attempt to inhibit the autoimmune response, thereby reducing cardiac inflammation (Figure 1). Mice received an intravenous injection of 7.5×10^7 naive, syngeneic splenocytes that had been coupled with cardiac myosin or BSA (sham control) with the cross-linking agent ECDI (day -7). One week later, the mice were immunized subcutaneously with 300 μg cardiac myosin in complete Freund’s adjuvant (day 0), followed by a boost 7 days later with the same antigen (day 7). At day 21, the effect of the tolerization protocol on the development of myocarditis and on myosin-specific immunity was analyzed by a number of methods: cardiac histology, DTH, and myosin-specific antibody production.

Myosin Tolerization Prevents the Development of Myosin-Induced Myocarditis

On day 21 postimmunization, hearts were excised for histological examination, and sections were examined after staining with hematoxylin and eosin and Masson’s trichrome (Figure 2). Myosin tolerization was highly effective at pre-
venting both the incidence and severity of myocarditis (Figure 2 and Table). Hearts from most of the myosin-tolerized mice (13 of 22) displayed no inflammatory cell infiltration or fibrosis (Figure 2A and 2E), comparable to heart tissue from PBS-immunized controls (Figure 2D and 2H). In 9 animals, tolerance was not complete, with 2 of 9 mice demonstrating severe myocarditis and 7 of 9 demonstrating mild inflammation (data not shown). This is in striking contrast to the hearts from sham-tolerized (Figure 2B and 2F) and nontolerized (Figure 2C and 2G) animals, which displayed massive mononuclear cell infiltration, myocyte swelling and necrosis, and fibrosis. All cardiac sections were scored for disease (scores ranged from 0, normal, to 3, most severe; see Methods for details), and the results are presented in the Table. The incidence of disease in the myosin-tolerized group was not significantly different from that of the PBS control group but was different from those of the sham (P < 0.002) and nontolerized (P < 0.003) groups. The myosin-tolerized mice (1.4/3.0) had a significantly reduced disease severity compared with sham (2.1/3.0, P < 0.02) and nontolerized (2.2/3.0, P < 0.002) mice, while not being significantly different from the PBS control group. Thus, myosin-specific tolerization was effective in decreasing disease incidence and severity in most of the mice that did develop disease.

**Inhibition of Myocarditis Through Antigen-Specific Tolerance Is Due to the Inhibition of Myosin-Specific Cellular Immunity**

We have hypothesized that this treatment induces antigen-specific T-cell anergy.\(^{20,21}\) Therefore, we analyzed T-cell immunity to the tolerizing antigen by measuring the myosin-specific DTH elicited in each treatment group (Figure 3). Myosin-specific DTH was significantly diminished in the myosin-tolerized mice (1.4/3.0) had a significantly reduced disease severity compared with sham (2.1/3.0, P < 0.02) and nontolerized (2.2/3.0, P < 0.002) mice, while not being significantly different from the PBS control group. Thus, myosin-specific tolerization was effective in decreasing disease incidence and severity in most of the mice that did develop disease.

**Antigen-Specific Tolerization Reduces Myosin-Specific Antibody Production in EAM**

An isotype-specific ELISA was developed to determine the effect of myosin tolerization on myosin-specific antibody production. Myosin-tolerized mice produced very little myosin-specific antibody of any isotype (Figure 4A). Although the antibody levels were higher than those detected in PBS controls (Figure 4D), they were much lower than those present in the sham-tolerized (Figure 4B) and nontolerized (Figure 4C) groups. Myosin-specific total IgG and IgG1 were

### Myosin Tolerization Reduces the Incidence and Severity of Myocarditis in Myosin-Immunized Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease Incidence* (% of All Mice)</th>
<th>Disease Severity,†</th>
<th>Disease Severity,†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin-tolerized</td>
<td>9/22 (41)‡</td>
<td>0.6 ± 0.19§</td>
<td>1.4 ± 0.29</td>
</tr>
<tr>
<td>Sham-tolerized</td>
<td>10/12 (83)</td>
<td>1.8 ± 0.35</td>
<td>2.1 ± 0.31</td>
</tr>
<tr>
<td>Nontolerized</td>
<td>12/13 (92)</td>
<td>2.0 ± 0.30</td>
<td>2.2 ± 0.27</td>
</tr>
<tr>
<td>PBS control</td>
<td>0/5 (0)</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Incidence includes all affected mice, regardless of disease severity.
†Mouse hearts were excised 21 days after myosin immunization and stained with hematoxylin and eosin or Masson's trichrome. Results are ± SEM.
‡Disease incidence in the myosin-tolerized group is significantly different from those of the sham-tolerized (P < 0.02) and nontolerized (P < 0.003) groups and not significantly different from the PBS control group.
§Scores for the myosin-tolerized group are significantly different from those of the sham-tolerized (P < 0.02) and nontolerized (P < 0.002) groups and not significantly different from the PBS control group. Scores for the sham-tolerized and nontolerized animals are not significantly different.
significantly lower in the myosin-tolerized mice than in the nontolerized (P<0.002 and 0.03, respectively) and sham-tolerized (P<0.0003 and 0.002, respectively) groups. None of the other antibody isotypes were significantly different in myosin-tolerized mice and control groups. Interestingly, the myosin-specific antibody isotypes produced by animals with myocarditis suggest that both Th1 (IgG2a)– and Th2 (IgG1)–type T-cell responses are present.22

Discussion
A wide variety of experimental methods of immunosuppression have been used to treat myocarditis and other autoimmune diseases, including administration of neutralizing antibody directed against T cells and antigen-presenting cells, such as anti-CD4,8,10 anti-CD8,8,10 and anti–major histocompatibility complex class II.23 Neutralization of proinflammatory cytokines such as tumor necrosis factor-α24 or trafficking molecules such as chemokines like macrophage inflammatory protein-1α25 have been used with some success. Although these are useful in downregulating damage due to autoimmunity, there may be adverse consequences in downregulating immune responses directed against an infectious agent.4 Conversely, treatments such as administration of recombinant interleukin-2 and interferon-α may aid in viral clearance but might promote autoimmune cardiac damage.3 All of these approaches suffer in part from a lack of specificity and do not overcome the challenges posed by infection-induced, organ-specific inflammatory diseases.

The goal of the present study was to inhibit only self-directed immune responses by restoring autoantigen-specific peripheral tolerance. Our tolerization protocol was highly successful in inhibiting cardiac inflammation induced by myosin immunization. Myofibrillar swelling and necrosis and reparative fibrosis were absent in the tolerized mice (Figure 2 and Table). Coupled-cell tolerance has been shown to downregulate both cellular and humoral immune responses as
measured by DTH and antibody production. In the Theiler’s murine encephalomyelitis virus model of multiple sclerosis, however, tolerance results in immune deviation in which the antibody isotypes produced switch from IgG2a and IgG2b, reflecting a Th1-type T-cell response, to IgG1, corresponding to a Th2-type response. In the present study, myosin-specific DTH was significantly decreased upon tolerization (Figure 3), and anti-myosin total IgG and IgG1 antibodies were significantly decreased (Figure 4). We performed all antibody analyses at 21 days after immunization, but future studies will also address responses during the entire disease course and when tolerance treatment is administered after disease onset. Studies in intravenous and nasal tolerization have also reported a decrease in antibodies directed against the tolerizing antigen, which is believed to be due to inhibition of T-cell help in tolerized animals. Although autoreactive B cells are present, autoantibody is not detected unless T-cell tolerance is overcome, as is true in EAM and is hypothesized to occur in human myocarditis. Clearly, autoantibodies to a number of cardiac antigens, including myosin, have been detected in humans with myocarditis, and it is possible that a pathogen or other damage-inducing event, such as myocardial infarction, could cause the breakdown in T-cell tolerance to self-cardiac antigens.

Among the possible mechanisms underlying peripheral tolerization are (1) direct anergy/deletion of Th1 lymphocytes; and (2) immune deviation, in which activation of Th2 lymphocytes occurs instead of the normal Th1 inflammatory cell activation; and (3) cytotoxic T-lymphocyte antigen 4 regulation of T-cell function by modulation of the threshold of T-cell activation, in which the strength of the signal by antigen stimulus must be much greater on cells expressing more molecules of cytotoxic T-lymphocyte antigen 4 or nonresponsiveness is maintained (T.N. Eagar et al, unpublished data, 2000). Unfortunately, it is not currently possible to analyze myosin-specific T-cell responses in vitro, because myosin is toxic to the cells and/or may induce their apoptosis (L.M.G., unpublished data, 2000). Our results support the theory that the treatment results in the antigen-specific nonresponsiveness of T cells, specifically Th1 T cells, responsible for the strong myosin-specific DTH. Myosin-specific antibody responses were also suppressed by the treatment, suggesting that tolerization also inhibits T-cell help for myosin-specific B-cell responses.

We chose the EAM model to evaluate the effectiveness of coupled-cell tolerance to treat myocarditis because it separates cardiac autoimmunity from the complicating immune responses directed against an infectious, disease-inducing agent. There are a number of murine models of myocarditis that can be used to study the efficacy of this tolerance protocol in the presence of an infectious agent. These models include protozoans, such as Trypanosoma cruzi, and viruses, such as coxsackieviruses and encephalomyocarditis virus. EAM and the infectious models are very similar in a number of
of ways, including the presence of myosin-specific autoantibodies, myocyte necrosis, and inflammatory cell infiltrate. It is interesting to note that strains of mice susceptible to coxsackievirus-induced myocarditis are also susceptible to cardiac myosin-induced myocarditis.2

In conclusion, antigen-specific peripheral tolerance induction provides a powerful tool for dissecting the mechanisms involved in cardiac autoimmunity. Although myosin-specific tolerization was used to prevent EAM in this report, it should be emphasized that tissue homogenates can be used as well.13 Thus, specific knowledge of the target proteins in an autoimmune response is not a prerequisite for administration of the therapy. It will be of interest to test the approach in other models of myocarditis, including Chagas disease and coxsackievirus myocarditis, and in cardiac allograft rejection. The results reported here indicate the usefulness of this method and the need to use its efficacy in infectious forms of myocarditis.

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