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
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 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 naïve mice and incubated at 37°C for 10 minutes in Tris-NH₄Cl (0.017 mol/L Tris free base, 0.14 mol/L NH₄Cl, 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 1500 g for 10 minutes in a 50-ml conical tube, and resuspended at a final concentration of 5x10⁷ 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 7.5x10⁷ 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 blind. 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 (Mitutoyo MTI Corp). Antigen (10 µg) in a 10-µL volume of DTH buffer (0.15 mol/L K₂HPO₄, 0.01 mol/L Na₃P₂O₇, 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.

**Serosogical 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 7.5x10⁷ naïve, 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 Tolerance 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-
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 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 animals compared with both the nontolerized (P < 0.0005) and sham-tolerized (P < 0.0005) groups. Responses were not significantly different between the myosin-tolerized and PBS control groups or between the sham-tolerized and nontolerized groups. These results indicate that T cells have been affected by the tolerization protocol in an antigen-specific manner and that the reduction in DTH, and disease, was not simply due to the introduction of ECDI-treated splenocytes in this model.

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
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-\(\alpha\)24 or trafficking molecules such as chemokines like macrophage inflammatory protein-1\(\alpha\)25 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-\(\alpha\) 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; (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. 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. 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 respects, and the findings from EAM should be true for analogous responses in myocarditis models.

Figure 4. Antigen-specific tolerization reduces myosin-specific antibody production in EAM. Levels of myosin-specific IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM and total IgG antibodies were assessed in each individual mouse serum by a cardiac myosin-specific ELISA. Total IgG, IgG1, IgG2a, and minimal IgG2b levels were analyzed in sham-tolerized (B) and nontolerized (C) animals. Myosin-tolerized mice produced very low levels of antibody on immunization with antigen (A), only slightly more than those produced by PBS controls (D). Levels of total IgG and IgG1 detected in sera of myosin-tolerized animals are significantly less than those in sera from nontolerized (P<0.002 and 0.03, respectively) and sham-tolerized (P<0.0003 and 0.002, respectively) groups. No other isotypes were significantly different between myosin-tolerized and nontolerized or sham-tolerized groups.
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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