Nasal Administration of Cardiac Myosin Suppresses Autoimmune Myocarditis in Mice

Yan Wang, MD, PhD, Marina Afanasyeva, MD, MPH, Susan L. Hill, DVM, Ziya Kaya, MD, Noel R. Rose, MD, PhD

Baltimore, Maryland

OBJECTIVES
This study was designed to examine whether myocarditis induced in a mouse model can be effectively suppressed by nasal administration of cardiac myosin (CM).

BACKGROUND
Myocarditis in humans often follows viral infection and is accompanied by evidence of an autoimmune response to CM. Treatment has been hampered by the fact that measures undertaken to reduce the autoimmune response often enhance the viral infection. Delivery of antigen via nasal route has been shown to induce antigen-specific tolerance and suppress certain autoimmune diseases in animal models.

METHODS
Myocarditis was induced in A/J mice by two subcutaneous injections of CM emulsified in complete Freund's adjuvant. Nasal instillation of CM (200 μg/mouse) or vehicle buffer was carried out three days before the first subcutaneous injection. The effect of nasal instillation of CM on cardiac histopathology, cytokine production by splenocytes, and antibody response was examined three weeks after the first subcutaneous injection.

RESULTS
Nasal administration of CM effectively reduced the severity of myocarditis. Consistent with the histological findings, the levels of interleukin-2 (IL-2), tumor necrosis factor-α, and IL-1β produced by splenocytes in response to CM were significantly decreased. In addition, the serum levels of IgE and IgG1 anti-myosin antibodies were suppressed. However, the levels of transforming growth factor-β (TGF-β) and CM-specific IgA antibodies were not affected.

CONCLUSIONS
Taken together, our results do not support active suppression through upregulation of TGF-β, IL-4, and IL-10 as a mechanism of tolerance, but favor anergy or deletion of both Th1 and Th2 autoreactive T cells. (J Am Coll Cardiol 2000;36:1992–9) © 2000 by the American College of Cardiology

Myocarditis in humans often follows infection by coxsackieviruses of group B3 and is accompanied by evidence of an autoimmune response to cardiac myosin (CM) (1). Efforts at treatment have been hampered by the fact that measures undertaken to reduce the autoimmune response often enhance the viral infection (2). An approach designed to achieve antigen-specific immunosuppression without impairing the anti-viral immunity would be beneficial. In a mouse model, we have shown that myocarditis can be induced by immunization with either murine cardiac myosin (MCM) or porcine cardiac myosin (PCM) (3,4). The cardiac lesions induced by MCM or PCM closely resemble those of human myocarditis. Myosin-induced myocarditis is a T-cell-dependent disease; however, antibodies have been shown to transfer the disease in some mouse strains (5–7). The role of Th1 and Th2 cytokines in the pathogenesis of autoimmune myocarditis is unclear, although tumor necrosis factor-α (TNF-α) has been implicated as an important mediator of disease (8,9).

Mucosal administration of antigens has been described as a method to induce antigen-specific tolerance and suppress autoimmune diseases in several animal models, including experimental autoimmune encephalomyelitis (EAE), experimental autoimmune myasthenia gravis (EAMG), experimental allergic uveoretinitis, insulin-dependent diabetes mellitus, and collagen-induced arthritis (10,11). Depending on the amount of antigen fed, the orally administered antigens can either induce regulatory cells that suppress the immune response through the local production of cytokines or can silence antigen-specific T cells by induction of clonal anergy or clonal deletion.

Recently, nasal administration of antigen has been reported as an alternative route to induce mucosal tolerance in some experimental autoimmune models (12–15). The nasal administration of antigen often appears to be more effective than oral administration in inducing tolerance (16). This difference can be explained by the fact that the antigen delivered via the nasal route is less likely to be degraded by acid and proteolytic enzymes that are present in the gastrointestinal environment. It was demonstrated that antigen given orally in milligram doses or nasally in microgram doses to Lewis rats prior to immunization with acetylcholine receptor (AChR) similarly prevented clinical signs of EAMG and suppressed AChR-specific B- and T-cell-mediated immune response (17).

In this study, we examined whether nasal administration of CM is effective in suppressing the induction of experimental autoimmune myocarditis in mice. We also investigated the effect of nasal instillation of CM on the cytokine...
Abbreviations and Acronyms

ACR = acetylcholine receptor  
CM = cardiac myosin  
CFA = complete Freund’s adjuvant  
ConA = concanavalin A  
EAE = experimental autoimmune encephalomyelitis  
EAMG = experimental autoimmune myasthenia gravis  
IFN-γ = interferon-γ  
IL-1β = interleukin-1β  
IL-2 = interleukin-2  
IL-4 = interleukin-4  
IL-10 = interleukin-10  
MCM = murine cardiac myosin  
PCM = porcine cardiac myosin  
TGF-β = transforming growth factor-β  
TNF-α = tumor necrosis factor-α

profiles and antibody response, and we discuss the possible mechanisms underlying the suppression of the disease.

METHODS

Induction of myocarditis. Myocarditis was induced in four- to five-week-old female A/J mice, which were obtained from the Jackson Laboratory (Bar Harbour, Maine) and maintained in the conventional animal facility at the Johns Hopkins School of Medicine. The PCM was extracted from pig ventricular muscle and MCM from pooled mouse hearts using the procedure as described previously (18). The purified CM was emulsified with equal volume of complete Freund’s adjuvant (CFA; Sigma, St. Louis, Missouri) supplemented with 5 mg/ml of Mycobacterium tuberculosis H37Ra. Each mouse was injected subcutaneously with 100 µl of the emulsion containing 200 µg of CM on day 0 and day 7. Control mice were given the same amount of emulsion containing CFA and vehicle buffer (0.5 mol/liter KCl, pH 6.8) without myosin. On day 0, each mouse containing the control mice also received an intraperitoneal injection of 500 ng of pertussis toxin (List Biological Laboratories, Campbell, California).

Nasal administration of antigen. Nasal administration of antigen was carried out by intranasal intubation three days prior to the first subcutaneous injection of CM. A fine, flexible plastic tubing (outside diameter 0.03 in. [0.08 cm] VWR), which was connected to a Hamilton syringe through a 27-gauge needle, was gently inserted 5 mm into a nostril of a mouse under ketamine anesthesia. Approximately 50 µl of CM or vehicle buffer (0.5 mol/liter KCl, pH 6.8) was slowly instilled into each mouse.

Histopathology. All mice were euthanized on day 21. The hearts were rapidly removed, fixed in 10% phosphate buffered formalin, and then embedded in paraffin. Serial sections were made through a portion of the heart. Every fifth section was placed on a slide and stained with hematoxylin and eosin. Evidence of myocarditis was determined blindly by two pathologists using a light microscopy according to a four-tier scoring system: Grade 1 corresponding to cardiac infiltration of approximately up to 10% of the cardiac sections; Grade 2, 11% to 20%; Grade 3, 21% to 40%; Grade 4, greater than 40%. The average score from the two pathologists’ readings was used for statistical analysis.

**T-Cell proliferation assay and in vitro cytokine production by splenocytes.** On day 21, the spleen of each mouse was teased into a single-cell suspension in RPMI-1640 (Life Technologies, Grand island, New York) using forceps. The splenic cells were sedimented and washed twice with 15 ml of fresh RPMI-1640 by centrifugation at 1,300 rpm (352 g) for 8 min. The viable cells were counted by trypan blue exclusion, and suspended at 1 x 10⁶ cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum (Life Technologies), 15 mmol/liter HEPES, 1% L-glutamine, 1% MEM vitamins, 1% nonessential amino acids, 0.1 mmol/liter β-mercaptoethanol, 1% sodium pyruvate, and 100 U/ml of penicillin-streptomycin. Then 100 µl/well of the cell suspension was dispensed into a 96-well U-bottom microtiter plate, which had already been loaded with 100 µl of the diluted antigen. Serial dilutions were made of CM ranging from 4.0 µg/ml to 0.25 µg/ml. Each plate also contained wells with medium alone or concanavalin A (ConA) as negative or positive controls, respectively. Each sample was tested in quadruplicate. The plates were incubated at 37°C in a humidified CO₂ incubator for 96 h, and then pulsed with 1 µCi/well of [³H]-thymidine (Amersham, Piscataway, New Jersey). After 18 h, the cells were harvested onto a piece of glass microfiber filter paper. The radioactivity was recorded using a Matrix-96 Direct beta-counter. The stimulation index was calculated by dividing the mean [³H]-thymidine incorporation of myosin-stimulated cultures by the mean [³H]-thymidine incorporation of cultures with medium alone.

For in vitro cytokine production, the viable splenocytes were cultured at 5 x 10⁶ well of a 24-well plate in RPMI-1640 complete medium in the presence of CM at 10 µg/ml. For each mouse, wells containing medium alone and ConA were also included as negative and positive controls. The plates were incubated at 37°C in a humidified CO₂ incubator. After 48 h, the supernatant was collected, aliquoted and frozen at −80°C for cytokine measurements as described below.

Quantitation of cytokines by ELISA. The levels of interleukins (IL)-2, IL-4, IL-10, IL-1β, interferon-γ (IFN-γ), TNF-α, and transforming growth factor-β (TGF-β) were measured by Quantikine cytokine ELISA kits (R & D Systems, Minneapolis, Minnesota) according to the manufacturer’s instructions. Each sample was tested in duplicate. The optical densities were obtained using an ELISA reader (Dynatech Laboratories, Chantilly, Virginia) at dual wavelengths of 450 nm and 570 nm. The quantity of each cytokine was calculated from the standard curve of the cytokine included on each plate. For every mouse, the level of each cytokine produced by splenocytes in the wells with medium alone was subtracted from that produced in response to myosin or ConA.
Detection of serum levels of anti-CM antibodies by ELISA. Flat-bottom 96-well microtiter plates were coated with 100 µl/well of CM at 5 µg/ml in bicarbonate buffer (pH 9.6). The plates were incubated in a damp box and incubated at 4°C overnight. The next day, the plates were blocked with 100 µl/well of phosphate-buffered saline (PBS), pH 7.2, containing 1% bovine serum albumin (BSA) and 0.05% Tween-20 (PBS/BSA/Tween), and incubated at 37°C for 1 h. After washing three times with PBS containing 0.05% Tween-20 (PBS/Tween), the plates were loaded with 100 µl/well of diluted serum samples. Each sample was tested in duplicate. The serum samples were diluted in PBS/BSA/Tween 1:400 for detection of total IgG antibodies, 1:200 for detection of IgG1, IgG2a, and IgG2b isotypes, and 1:50 for IgA antibodies. Each plate contained the same positive and negative control serum samples. The plates were incubated with sera overnight at 4°C in a damp box. On the third day, the plates were washed three times with PBS/Tween and incubated at 37°C for 1 h with 100 µl/well of peroxidase-conjugated goat anti-mouse secondary antibodies diluted to 1:1000 for IgG (KPL, Gaithersburg, Maryland), 1:500 for IgG1, IgG2a (PharMingen, San Diego, California), IgG2b (Bethyl, Montgomery, Texas) and IgA (Sigma, St. Louis, Missouri). Finally, after three additional washes with PBS/Tween, plates were loaded with 100 µl/well of 3,3′,5,5′-tetramethylbenzidine substrate solution and incubated at room temperature for about 5 to 15 min. The color reaction was stopped by adding 100 µl of 1 mol/liter sulfuric acid. The optical density (OD) values were determined by an ELISA reader at 450 nm wavelength. Results were expressed as the adjusted ODs [=[Sample OD reading × 100/(OD of positive control − OD of negative control)]. Serum concentrations of total IgE were determined using anti-mouse IgE capture and detection antibodies (PharMingen, San Diego, California) according to the protocol recommended by the manufacturer. The concentration of IgE of each testing sample was read off the IgE standard curve.

Statistical analysis. Statistical differences in the levels of cytokines and antibodies were determined using the Mann-Whitney U test. A p value of < 0.05 was considered significant.

RESULTS

Intranasal administration of CM reduced the severity of autoimmune myocarditis. Autoimmune myocarditis was induced in A/J mice by two subcutaneous immunizations on days 0 and 7 with either PCM or MCM in CFA. To determine whether nasal administration of CM can prevent the induction of myocarditis, we first administered 200 µg/mouse of PCM intranasally to A/J mice either once on day −3, twice on days −3, and +4, or three times on days −3, +4, and +12. We found that a single nasal instillation of PCM three days prior to the first immunization (day −3) effectively reduced the severity of myocarditis on day 21 (Fig. 1A), as compared with the positive control mice that received vehicle buffer intranasally. In the positive control group, 6 of 14 mice developed myocarditis. The severity of disease of the six mice ranged from 1 to 4, with an average of 2.3 (Fig. 1B). In the treatment group, only one of nine mice was found to have a small focal cardiac infiltrate, which was less than 5% of the total heart section (Fig. 1C). Two and three nasal instillations did not appear to be more effective than a single administration in suppressing the induction of the disease.

The experiment was repeated using MCM instead of PCM for both nasal instillation and subcutaneous immunization. Nasal administration of MCM also significantly reduced the severity of myocarditis on day 21 (p = 0.048, Fig. 1A). In the positive control group of 10 mice that received vehicle buffer intranasally, 6 developed severe myocarditis with more than 30% of the heart infiltrated (grades 3−4), and the rest developed mild to moderate disease. The average disease severity was 3.0. However, in the treatment group of nine mice that received MCM intranasally, only one developed severe myocarditis (grade 4); five developed mild to moderate disease; and three had no disease. The average disease severity was 1.0. Thus, nasal instillation of cardiac myosin can effectively reduced the severity of autoimmune myocarditis in mice.

Intranasal administration of CM suppressed the production of IL-2, TNF-α and IL-1β by splenocytes. To examine the changes in cytokine profile in response to intranasal administration of CM, splenocytes were collected on day 21 from mice that were treated intranasally with PCM or that were treated with vehicle buffer, and cultured in vitro in the presence of PCM or ConA. The levels of various cytokines produced in the culture supernatants including IL-2, IFN-γ, IL-4, IL-10, TNF-α, IL-1β, and TGF-β were then measured. All of these cytokines were detectable in the supernatants of splenocytes cultured in the presence of PCM, with the levels of IFN-γ being the highest, IL-2, TNF-α, and TGF-β being the moderate, and IL-10, IL-4, and IL-1β being the lowest. As shown in Figure 2, mice that were treated with PCM intranasally generated significantly lower levels of IL-2 in response to PCM than did control mice treated intranasally with buffer (p = 0.048). However, the levels of IL-2 produced in response to ConA were the same in mice that received PCM intranasally and that received buffer intranasally, indicating there was no general suppression of the immune response (Fig. 2).

In mice that received PCM intranasally, the levels of TNF-α (p = 0.04) and IL-1β (p = 0.02) produced in response to PCM were also significantly reduced, in comparison with control mice receiving buffer intranasally (Fig. 2). Although the levels of other cytokines such as IFN-γ and IL-10 were also lower in mice that received PCM intranasally, the decrease was not found to be statistically significant. In addition, there was no apparent difference in the levels of IL-4 (data not shown) and TGF-β (Fig. 3).
between control mice that received buffer intranasally and mice that received CM intranasally.

In the parallel experiment where MCM was used instead of PCM for nasal instillation and subcutaneous immunization, the levels of aforementioned cytokines were also measured and compared between mice that were treated intranasally with and without MCM. All the cytokines listed above except IL-1β and IL-4 were detected, and the overall levels of detectable cytokines were generally lower than those detected in the experiment where PCM was used as antigen. Nevertheless, in line with the above findings, the levels of IL-2 and TNF-α were markedly decreased in mice that were treated with MCM intranasally compared with those that received vehicle buffer intranasally. Therefore, nasal instillation of CM had caused a significant decrease in the levels of IL-2, TNF-α, and IL-1β, correlating with the suppression of the disease.

We also examined the effect of nasal instillation of cardiac myosin on T-cell proliferative response. Splenocytes were collected on day 21 and cultured in the presence of varying doses of cardiac myosin or ConA. In the positive control mice that received buffer intranasally, it was found that the average stimulation index to CM was about 2 to 3 and the average stimulation index to ConA was about 25. In mice that received CM intranasally, the stimulation indices to CM and ConA were similar to those of the positive control
mice. Thus, nasal instillation of CM did not suppress T-cell proliferation to either CM or ConA.

**Intranasal administration of CM caused a decrease in the serum levels of IgE and anti-myosin antibodies of IgG1 subclass.** To investigate the effect of nasal instillation of cardiac myosin on the humoral response, the serum levels of IgE and PCM-specific antibodies including IgA, IgG, and its subclasses IgG1, IgG2a and IgG2b were measured. Sera were collected on day 10 and day 21 to measure antibody levels postimmunization. Levels of the antibodies were compared between mice that received PCM intranasally and those that received buffer intranasally. As shown in Figure 4, mice that received PCM intranasally had markedly lower levels of IgE on both day 10 and day 21 than mice that received buffer intranasally. The decrease in IgE was more pronounced on day 10 (p = 0.01). Moreover, the levels of PCM-specific IgG1 antibodies on both day 10 (p = 0.04) and day 21 (p = 0.04) were significantly reduced in mice that received PCM intranasally, compared to those that received buffer intranasally (Fig. 5). Although the levels of IgG, IgG2a, and IgG2b anti-PCM antibodies were also slightly lower in mice that were treated intranasally with PCM, the decrease was not statistically significant. Furthermore, the levels of PCM-specific IgA antibodies were comparable between mice that received PCM nasally and those that received buffer nasally. Thus, nasal instillation of CM significantly reduced the serum levels of IgE and myosin-specific IgG1 antibodies, correlating with disease suppression.

**DISCUSSION**

In the present study, our results demonstrate that CM administered intranasally to A/J mice can effectively sup-
press the induction of murine autoimmune myocarditis. The suppression of disease correlated with significant reduction in the levels of IL-2, TNF-\(\alpha\) and IL-1\(\beta\), and in the serum levels of IgE and IgG1 anti-myosin antibodies. No increase was observed in the levels of TGF-\(\beta\), and serum IgA anti-myosin antibodies.

Possible mechanisms that underlie the suppression of disease. The mechanisms of mucosal tolerance have been extensively studied in other autoimmune disease models, particularly in EAE. Two major mechanisms have been proposed based on the dose of the antigen administered orally (10,11). Repeated low doses of antigen favor the generation of regulatory cells secreting cytokines, such as TGF-\(\beta\), IL-4 and IL-10, which antagonize the development of a Th1 response. In contrast, a single high dose of antigen causes the deletion or anergy of both Th1 and Th2 autoreactive T cells. Recent studies on nasal tolerance in autoimmune models, particularly in EAMG, suggested that tolerance could be induced via similar mechanisms (19).

To understand the possible mechanisms that underlie the suppression of autoimmune myocarditis, we examined the effect of nasal administration of CM on the balance of Th1 (IL-2, IFN-\(\gamma\)) and Th2 (IL-4, IL-10) cytokines, on the production of TGF-\(\beta\), TNF-\(\alpha\), and IL-1\(\beta\), and on the antibody response. Our results showed that the levels of IL-2, a prototypic Th1 cytokine, were significantly lower in mice that received myosin intranasally compared to the control mice that received buffer intranasally. The reduction in the levels of IL-2 was specific to CM stimulation because no decrease was observed upon ConA stimulation. When we measured the serum levels of IgE and myosin-specific IgG1 antibodies, a significant decrease occurred in mice that received myosin intranasally as compared to the control mice receiving buffer intranasally. Because the isotype switch to IgE and IgG1 is known to be driven by IL-4 (20,21), the reduction in IgE and IgG1 anti-myosin antibodies in mice that received myosin intranasally may indicate the downregulation of IL-4, which is the prototypic Th2 cytokine. We were unable to show a significant decrease in the levels of IL-4 produced by splenocytes. This may be due to the fact that the levels of IL-4 were at the limit of detection and the method used was not sensitive enough to demonstrate the reduction.

In addition, we also observed a significant reduction in the levels of TNF-\(\alpha\) and IL-1\(\beta\) produced by splenocytes from mice that received intranasal instillation of CM. This is consistent with our previous finding that high levels of TNF-\(\alpha\) are strongly associated with the development of myocarditis upon immunization with CM (4). Furthermore, it is interesting to mention that TNF-\(\alpha\) is a cytokine

![Figure 3.](image1.png) Nasal administration of CM did not reduce the production of TGF-\(\beta\) by splenocytes in response to CM. The splenocytes from mice that received either CM (right bar, \(n = 4\)) or vehicle buffer (left bar, \(n = 3\)) intranasally were cultured in vitro in the presence of CM (10 \(\mu\)g/ml). The levels of TGF-\(\beta\) produced in the culture supernatant were measured and presented as the mean of each group \(\pm\) SEM. There was no decrease in the levels of TGF-\(\beta\) observed in mice that received CM nasally as compared to those that received buffer nasally.

![Figure 4.](image2.png) Nasal administration of CM reduced the serum levels of total IgE antibodies. Sera were collected on days 10 and 21 from mice that received CM intranasally (right bars, \(n = 9\)) and those that received vehicle buffer (left bars, \(n = 14\)). The serum levels of total IgE antibodies (ng/ml) were measured by a capture ELISA and presented as the mean of each group \(\pm\) SEM. The levels of IgE were lower in mice that received CM intranasally on both days 10 and 21, compared to those that received buffer. The decrease in IgE levels on day 10 was statistically significant (\(p = 0.01\)).
that can be produced during both Th1 and Th2 immune responses (22). Thus, these results do not favor a mechanism of immune deviation from Th1 response to Th2 response, but rather suggest clonal anergy or deletion of both Th1 and Th2 autoreactive T cells. At present, we do not know if this effect is dose-related. Future studies of applying multiple lower doses of CM intranasally would be useful. Despite the reduced production of cytokines by spleen cells, we did not detect a significant decrease in T-cell proliferation to varying doses of CM in mice that received CM intranasally. This may be attributed to the likelihood that T-cell proliferative response is not a sensitive measure of T-cell activation in our model. This is supported by the observation that splenic T cells from myocarditic mice can transfer disease to irradiated naïve mice (unpublished results) and produce high levels of various cytokines when stimulated in vitro with CM, even though they do not proliferate well to the autoantigen. The disease development is much better correlated with cytokine production rather than T-cell proliferation to CM (4).

**The role of TGF-β in the suppression of disease.** The role of TGF-β in mediating mucosal tolerance is controversial. Evidence shows that orally administered antigen can generate populations of cells secreting TGF-β in the gut-associated lymphoid tissue, and these cells are capable of regulating the development of Th1 responses (10). It has also been shown that in vivo blocking of TGF-β by anti-TGF-β antibodies abolished the tolerance induction by nasal administration of AChRs in murine model of myasthenia gravis (23). However, the role of TGF-β in mediating oral tolerance has recently been challenged by the report of successful induction of oral tolerance in TGF-β null mice (24), indicating an alternative mechanism of tolerance. In our study, we did not observe any increase in the levels of TGF-β in mice that received CM intranasally.

Our findings that the serum levels of myosin-specific IgA antibodies were not elevated in mice that received myosin nasally further argue against the role of TGF-β in the induction of nasal tolerance, because TGF-β is known to be a switch factor for IgA production (25). Taken together, our results do not support active suppression through production of TGF-β, IL-4, and IL-10 as a mechanism of tolerance, but favor anergy or deletion of both Th1 and Th2 autoreactive T cells.

**Relevance to human myocarditis.** The appropriate treatment of myocarditis in humans remains a major clinical problem (2). In general, immunosuppressive therapy is not effective, although some patients clearly benefit from it. These conflicting results may be attributed to the likelihood that many cases represent a mixture of virus-induced and immune-mediated myocarditis. An approach designed to reduce the immunopathological effects without impairing the development of anti-viral immunity would be beneficial in treating this or other infection-associated autoimmune disorders. The present evidence shows that the disease can be specifically reduced by nasal administration of antigen before the onset of the autoimmune disease without impairing the general immune response. Future studies will be directed to nasal treatment instituted after immunization.

In addition, studies are underway to test the effect of nasal treatment with cardiac myosin on CB3 virus-induced chronic myocarditis in mice. Because other heart antigens, such as adenine nucleotide translocator, branched chain ketoacid dehydrogenase, sarcoplasmic reticulum calcium ATPase, and laminin have also been implicated in experimental as well as clinical myocarditis (26–29), it would be interesting to study whether nasal administration of those autoantigens has any impact on viral myocarditis.
REFERENCES


