Cardiac alpha-myosin heavy chains differ in their induction of myocarditis. Identification of pathogenic epitopes.

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Research Article

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Cardiac α-Myosin Heavy Chains Differ in Their Induction of Myocarditis

Identification of Pathogenic Epitopes

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Abstract

BALB/c mice develop autoimmune myocarditis after immunization with mouse cardiac myosin, whereas C57B/6 mice do not. To define the immunogenicity and pathogenicity of cardiac myosin in BALB/c mice, we immunized mice with different forms of cardiac myosin. These studies demonstrate the discordance of immunogenicity and pathogenicity of myosin heavy chains. The cardiac α-myosin heavy chains of BALB/c and C57B/6 mice differ by two residues that are near the junction of the head and rod in the S2 fragment of myosin. Myosin preparations from both strains are immunogenic in susceptible BALB/c as well as in nonsusceptible C57B/6 mice; however, BALB/c myosin induces a greater incidence of disease. To further delineate epitopes of myosin heavy chain responsible for immunogenicity and disease, mice were immunized with fragments of genetically engineered rat α cardiac myosin. Epitopes in the region of difference between BALB/c and C57B/6 (residues 735–1032) induce disease in both susceptible and nonsusceptible mice.

The data presented here demonstrate that pathogenic epitopes of both mouse and rat myosin reside in the polymorphic region of the S2 subunit. In addition, these studies suggest that polymorphisms in the autoantigen may be part of the genetic basis for autoimmune myocarditis. (*J. Clin. Invest.* 1993. 90:2877–2882.) Key words: autoimmunity • cardiology • lymphocytes • myocarditis • myocardium

Introduction

Experimental murine autoimmune myocarditis can be triggered by Coxsackie B3 virus (CB3) infection (1) and susceptibility to the virus-induced autoimmune disease is under genetic control. BALB/c mice, for example, develop autoimmune myocarditis after CB3 infection, whereas C57B/6 mice do not (2–4). Susceptible mice develop a chronic inflammatory cell infiltrate and myocyte necrosis associated with heart-specific autoantibodies and autoreactive T cells (5–13). A major autoantigen is cardiac myosin heavy chain (Myhc)[1] (10, 11). Mice susceptible to CB3-induced autoimmune myocarditis will develop a myocarditis that is similar immunologically and histologically to the post-viral myocarditis after immunization with mouse cardiac myosin and mice that do not develop autoimmune myocarditis after CB3 infection also do not develop myocarditis after immunization with cardiac myosin (13).

Traystman et al. (14) have shown that susceptibility to CB3-induced myocarditis in mice maps to chromosome 14 near the loci for the T cell receptor α chain and the α and β Myhc. Myhcα is the predominant Myhc expressed in the adult rodent myocardium (15). Recent sequence studies of murine strains show Myhcα allelic polymorphism (16). Myhcα from BALB/c mice, a strain susceptible to both viral and myosin-induced myocarditis, differs at 2 of 1938 amino acid residues (838 and 955) from the Myhca from myocarditis-resistant C57B/6 mice. While previous studies of myosin-induced myocarditis have not controlled for the strain from which cardiac myosin was purified, these data now raise the possibility that allelic polymorphism in Myhcα may contribute to the genetic basis for susceptibility to autoimmune myocarditis and provide an opportunity to study the importance of particular polymorphic regions of myosin in the induction of autoimmune myocarditis. The present study was therefore, undertaken to determine whether sequence differences in Myhcα alter the immune response to cardiac myosin and to delineate immunogenic and pathogenic regions of cardiac myosin. The results demonstrate that some immunogenic epitopes of cardiac myosin reside in the myosin head and that pathogenic epitopes of both mouse and rat Myhcα reside in the region from amino acids 736 to 1032. Furthermore, the polymorphic residues 838 and 955 in the S2 subunit of mouse Myhcα may be important in the induction of myocarditis in BALB/c mice suggesting that polymorphisms in myosin genes may constitute part of a genetic susceptibility to disease.

Methods

Animals. 4–6-wk-old female BALB/c and C57B/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained at the Albert Einstein College of Medicine animal facility.

Mouse cardiac myosin preparation. Mouse cardiac myosin was purified from either BALB/c or C57B/6 hearts according to the method of Pollack et al. (17). The myosin concentration was determined spectrophotometrically using an extinction coefficient of 5.4.

Expression of genetically engineered myosin fragments. The cDNAs for rat D0 and D4 Myhcα fragments were cloned into the pKK 233-2 vector (Pharmacia, Inc., Piscataway, NJ) with a hybrid trp-lac promoter inducible with isopropyl-β-D-thiogalactopyranoside and an initiation codon contained within an NcoI site (18). The JM105 strain of *Escherichia coli* was transformed with the recombinant expression plasmids and grown in 1 liter of Luria-Bertani broth to an OD of 0.7. The cells were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 35–40 min. A cell pellet was washed in 120 ml of a low salt buffer (10 mM triethanolamine, pH 8 at 4°C, 0.5 mM ATP, 0.5 mM DTT, 0.1 mM $CaCl_2$) and resuspended in 20 ml of low salt buffer containing protease inhibitors (aprotinin 20 μg/ml, leupeptin 10 μg/

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1. *Abbreviations used in this paper:* IFA, incomplete Freund's adjuvant; Myhc, cardiac myosin heavy chain.

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ml, pepstatin 2.5 μg/ml, and chymostatin 2.5 μg/ml). Bacteria were lysed by exposure to 100 lbs/in^2 pressure in a precooled French pressure lysis cell. A pellet was obtained by centrifugation and was resuspended in 10 ml of Sarkosyl extraction buffer (10 mM triethanolamine, 4 mM ATP, 0.8 mM DTT, 1 mM EDTA, 0.5% Sarkosyl, and the above protease inhibitors plus 0.5 mM phenanthroline) and then centrifuged at 30,000 *g* for 1 h. The supernatant was electrophoresed on a 10% SDS-polyacrylamide gel and the protein was electroeluted from the appropriate band. Generally, 1 liter of starting culture yielded 1.2–1.5 mg of pure protein.

Induction of myocarditis. A total of 100 μg of myosin or Myhcα fragment was emulsified in CFA (DIFCO Laboratories, Detroit, MI), and female BALB/c or C57B/6 mice were injected subcutaneously at four sites. 1 wk later, the mice were boosted s.c. with 100 μg of myosin or recombinant fragments in CFA. A second immunization protocol consisted of injecting i.p. 500 ng of pertussis toxin (kindly provided by Sigma Chemical Co., St. Louis, MO) at the time of the first immunization (13). Mice given pertussis toxin were boosted with myosin in incomplete Freund's adjuvant (IFA, DIFCO Laboratories) on weeks 2, 4, and 6. Mice were killed after 8 wk and the hearts were rapidly excised and put in 10% buffered formalin for histological study.

Serum anti-myosin reactivity. Sera from immunized mice were tested for IgG antibodies to mouse cardiac myosin by ELISA as described by Neu et al. (13). Briefly, myosin was adsorbed to microtiter plates (Falcon Labware, Lincoln Park, NJ), blocked with PBS-2% BSA (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and then incubated with serial dilutions of sera from immunized mice. Anti-myosin antibodies were detected with peroxidase conjugated anti-mouse IgG diluted 1:1000 (Sigma Chemical Co.) and substrate, 2, 2′-, azinodi-3-ethylbenzthiazoline sulfonate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Optical density was read at 405 nm on an ELISA reader.

Histology. Formalin-fixed hearts were embedded in paraffin according to standard procedures. Sections were prepared from hearts dissected in the atrial-apical axis thereby producing two halves in which all four chambers could be seen. Three levels, each ~ 5 μm in thickness, were prepared and stained with hematoxylin-eosin. Sections were evaluated from all hearts by a cardiac pathologist (Dr. Factor), who was blinded to the strain and immunization status of each animal. Each heart section was examined for active myocarditis and its extent and localization if present. The diagnosis of myocarditis was according to the Dallas criteria (19). The major criteria that establish the diagnosis of myocarditis include an inflammatory infiltrate with associated myocyte necrosis or damage. The presence of interstitial myocardial inflammation without necrosis, so-called borderline myocarditis (19), was also noted.

Results

Anti-myosin antibodies in mice immunized with mouse cardiac myosin or rat Myhcα fragments. Both BALB/c and C57B/6 mice immunized with mouse cardiac myosin, whether of BALB/c or C57B/6 origin, developed elevated titers of IgG anti-myosin antibodies (Fig. 1, *top*). C57B/6 mice developed equivalent anti-myosin titers independent of the origin of the myosin whereas BALB/c mice generated slightly higher titered reactivity to C57B/6 myosin than to autologous BALB/c myosin. There was no difference in antibody titer in those mice that developed disease and those that did not.

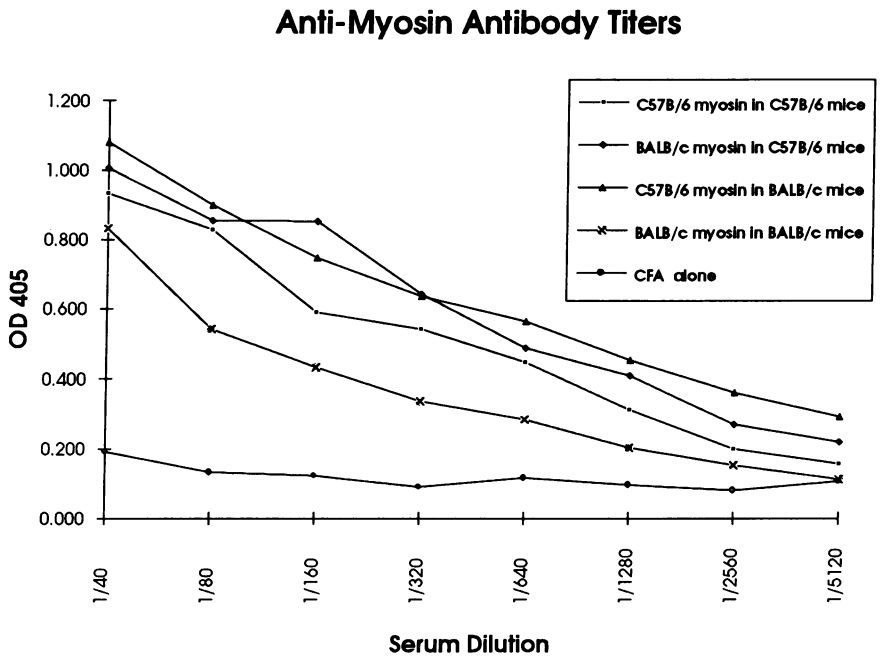
Both BALB/c and C57B/6 mice immunized with fragments of rat Myhcα developed elevated titers of anti-myosin antibodies (Fig. 1, *bottom*). Titers were slightly lower in these mice than in mice immunized with intact mouse myosin, presumably because there are additional immunogenic epitopes in the rod region. The observation that mice immunized with the D0 or D4 fragment of rat Myhcα develop anti-myosin antibodies demonstrates their immunogenicity in both mouse strains.

BALB/c cardiac myosin is more efficient at inducing myocarditis than C57B/6 cardiac myosin. In order to study whether the allelic differences between BALB/c and C57B/6 myosin contribute to the differential susceptibility to myosin-induced myocarditis seen in these two strains, mice were immunized with either BALB/c or C57B/6 myosin using two immunization protocols. The incidence of myocarditis in immunized BALB/c and C57B/6 mice is shown in Table I and confirms the susceptibility of BALB/c mice shown in previous studies (13). When mice were immunized with myosin in CFA and then boosted with the same, all 10 BALB/c mice immunized with BALB/c myosin developed myocarditis (Fig. 2, *top*) and only 5 of 10 BALB/c mice immunized with C57B/6 myosin showed evidence of myocarditis (Fig. 2, *middle*). In contrast only 1 of 10 C57B/6 mice immunized with either BALB/c or C57B/6 myosin in CFA developed autoimmune myocarditis. When mice were given pertussis toxin at the time of the initial immunization and boosted with myosin in IFA, 3 of 5 BALB/c mice receiving BALB/c myosin developed myocarditis, while none of 5 BALB/c mice receiving C57B/6 myosin developed myocarditis. CFA alone did not induce myocarditis either in the presence or absence of pertussis toxin. Our results suggest that BALB/c myosin is more pathogenic than C57B/6 myosin in the susceptible BALB/c mice. As BALB/c and C57B/6 myosin differ only at residues 838 and 955, these residues must contribute to disease induction.

Pathogenic epitope(s) of rat myosin reside in the S2 subfragment from amino acid 736 to 1032. To further explore regions of Myhcα involved in pathogenesis of autoimmune myocarditis in mice and to determine whether myosin polymorphisms cause myocarditis, mice were immunized with genetically engineered fragments of rat Myhcα produced in and purified from *E. coli* (Fig. 3). Fragment D0 encodes amino acids 1–735 and fragment D4 encodes amino acids 1–1032. Rat D0 differs from the analogous mouse residues at seven positions while D4 differs by five and four additional residues from C57B/6 and BALB/c myosin, respectively. Table II displays the incidence of myocarditis seen in mice immunized with these fragments. None of five BALB/c mice immunized with fragment D0 developed histologically evident disease, whereas all five BALB/c mice immunized with D4 developed foci of myocarditis. To determine whether the rat D4 fragment would also be pathogenic in a nonsusceptible strain, we immunized C57B/6 mice with D4. Three of 5 C57B/6 mice developed myocarditis (Fig. 2, *bottom*). Since D4 is longer than D0 by residues 736–1032, the region spanning these residues contains one or more pathogenic epitope(s) that can cause disease in both a susceptible and a nonsusceptible mouse strain.

Discussion

Mice of different genetic backgrounds have different predispositions to autoimmune disease. While genes in the MHC have been implicated in disease susceptibility, it is clear that susceptibility is multigenic (2, 4). One gene associated with susceptibility to murine autoimmune myocarditis localizes to chromosome 14 (14). The locus for the α chain of the T cell receptor is located on this chromosome and could well be a locus important in determining disease susceptibility. In addition, the locus for Myhcα maps to chromosome 14 (14). Recently, it has shown that there are several alleles of mouse Myhcα (16) raising the possibility that polymorphisms of the targetted auto-



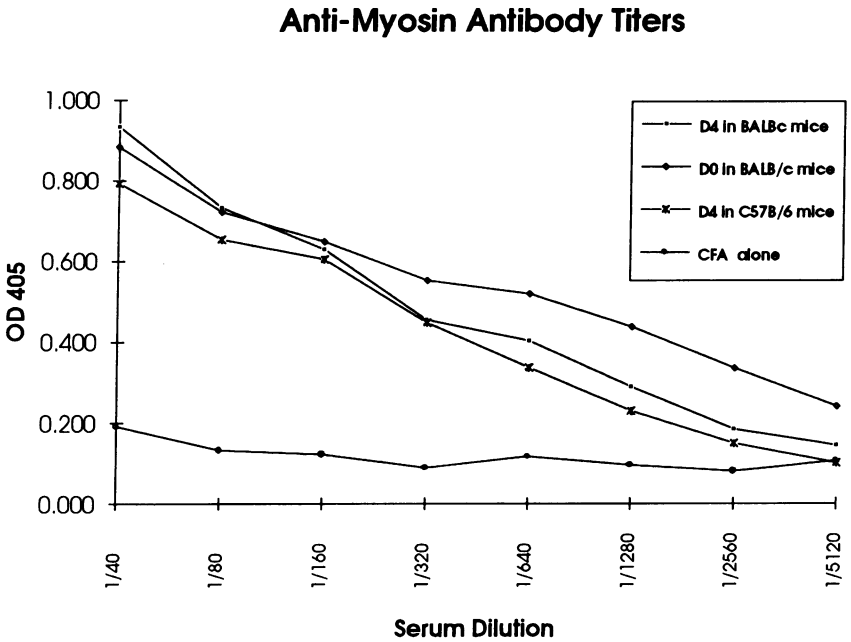


Figure 1. Anti–cardiac myosin antibodies in sera. BALB/c or C57B/6 mice were immunized with (*top*) BALB/c or C57B/6 myosin or (*bottom*) genetically engineered fragments rat Myhcα D0 or D4 emulsified in CFA and were boosted with the same immunogen 7 d later. Serum samples were obtained 2–3 wk after the first immunization. Anti–myosin antibodies were assayed by ELISA. Each line represents an average of five mice.

antigen could influence susceptibility to autoimmune myocarditis.

In this study, we attempted for the first time to study allelic forms of murine cardiac myosin for their ability to induce both an antibody response and autoimmune disease. In addition, we used defined mouse myosins and fragments of rat Myhcα to further delineate pathogenic epitopes of myosin. We analyzed responses in both BALB/c mice, a strain known to be susceptible to CB3-induced and myosin-induced autoimmune myocarditis as well as C57B/6 mice, a strain which is resistant to CB3-induced and mouse myosin-induced myocarditis.

Our data suggest that allelic polymorphisms in Myhcα contribute to disease pathogenesis. Both BALB/c and C57B/6 myosin were immunogenic in BALB/c mice, since immunized mice developed high titers of anti–myosin antibodies; however, BALB/c myosin induced a significantly higher incidence of myocarditis than C57B/6 myosin. In contrast to other studies that have shown low anti–myosin antibody titers in nonsusceptible mice immunized with myosin (2, 11), we now demonstrate that C57B/6 mice develop anti–myosin antibodies in equal titer to those made in BALB/c mice after immunization with either BALB/c or C57B/6 myosin. The C57B/6 mice,

Table I. Induction of Autoimmune Myocarditis in BALB/c Mice by Alleles of Murine Myosin

Myosin	Myocarditis recipient strain	
	BALB/c	C57B/6
Experiment 1		
BALB/c*	10/10	1/5
C57B/6	5/10	0/5
Experiment 2		
BALB/c‡	3/5	
C57B/6	0/5	

BALB/c or C57B/6 mice were immunized with BALB/c myosin or C57B/6 myosin using immunization protocols described in Methods. Mice in experiment 1 were immunuized with myosin in CFA and boosted with the same. Mice in experiment 2 were immunized with myosin in CFA and given pertussis toxin at the time of the initial immunization. They were boosted with myosin in IFA. The heart was excised from each mouse at 8 wk and fixed in 10% buffered formalin. Hearts were sectioned and stained with hematoxylin-eosin. * $P <$ 0.05. ‡ $P < 0.05$.

however, did not develop significant myocarditis with either preparation. Interestingly, BALB/c mice developed slightly higher titers of anti-myosin antibodies when immunized with C57B/6 myosin than with BALB/c myosin despite developing disease more readily after immunization with autologous myosin. These data confirm previous reports showing that anti-myosin antibodies need not cause cardiac damage and do not significantly contribute to the myocarditis of BALB/c mice (20). A similar inverse relationship between antibody titer and disease induction has been reported for experimental autoimmune encephalomyelitis (21).

Myosin induces a helper T cell and a B cell response in both mouse strains. Its immunogenicity is independent of polymorphisms at residues 838 and 955. Each strain can clearly process myosin and present some fragments of both myosin alleles to T cells. It is possible that the pathogenicity of BALB/c myosin in BALB/c hosts reflects a difference in processing and presentation of Myhcα peptide fragments by antigen presenting cells that, in ways yet to be elucidated, activates T cells making a particular panel of cytokines that can cause direct cardiac injury. The two alleles differ by only two amino acids, 838 and 955, both in the subfragment 2 region of the molecule. This region of the molecule is sensitive to protease digestion in vitro. It may be that these amino acid differences alter proteolytic degradation of myosin in vivo or perhaps affect the interaction of the proteolytically generated peptide fragments with class II molecules.

In this study, we show that antibodies can be elicited by and are reactive to the myosin head (fragment D0). Although mice immunized with D0 develop anti-myosin antibodies, they do not develop disease. While seven amino acid residues distinguish the rat fragment D0 from the homologous region of BALB/c and C57B/6 myosin, we predict that the homologous fragment from mouse Myhcα will also be nonpathogenic. It may be that there are inadequate numbers of T and B cells reactive with this region, or alternatively, the autoreactive lymphocytes may not mediate cardiac damage.

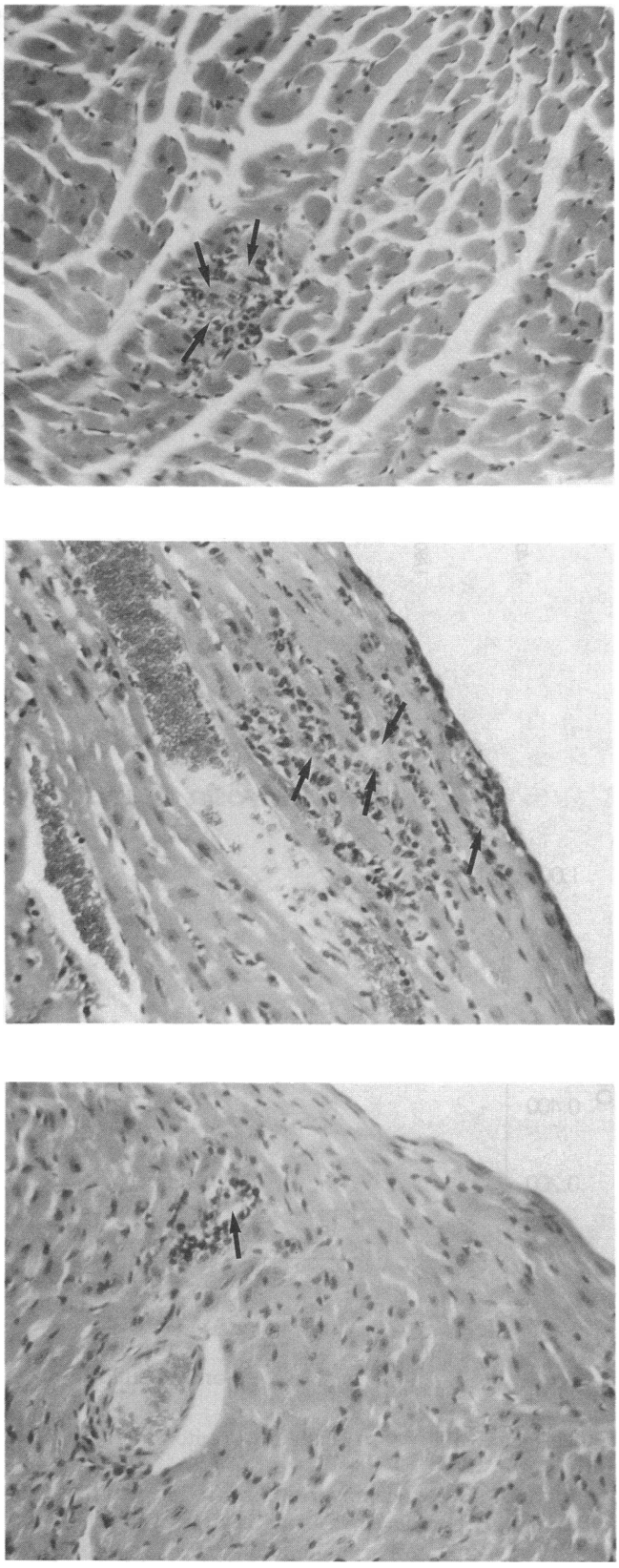
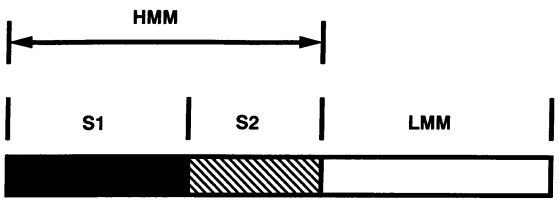


Figure 2. (*Top*) BALB/c mouse immunized with BALB/c myosin. There is a discrete focus of myocarditis in the left ventricular free wall. Several necrotic and degenerating myocytes (*arrows*) are surrounded by numerous mononuclear inflammatory cells. Hematoxylin-eosin, ×320. (*Middle*) BALB/c mouse immunized with



Residue	741	806	838	901	955
BALB/c	R	I	I	N	I
C57B/6	-	-	S	-	N
Rat Myhca (736-1032)	G	M	-	A	D

Figure 3. Diagram of mouse myosin heavy chain and the homologous fragments of rat myosin. Amino acid differences distinguishing BALB/c from C57B/6 myosin and rat myosin for the region of D4 that spans residues 736–1032 are shown. Amino acids are shown relative to the BALB/c sequence.

The D4 fragment of rat Myhcα is both immunogenic and pathogenic. This region of rat Myhcα differs from BALB/c Myhcα by four amino acids and from C57B/6 myosin by five amino acids and includes the region of polymorphism between BALB/c and C57B/6 Myhcα. Rat Myhcα is identical to BALB/c Myhcα at residue 838 and different from both mouse strains at residue 955. The D4 fragment is pathogenic in both susceptible BALB/c mice and nonsusceptible C57B/6 mice. It is likely that residues 741, 806, 901 and/or 955 are responsible for the increased pathogenicity of this rat Myhcα fragment in mice.

The immunogenicity of an autoantigen is clearly distinct from its pathogenicity. The data presented here show that polymorphisms in autoantigen can influence pathogenicity. Some pathogenic epitopes reside in the region spanning residues 736–1032 and residues 838 and 955 which differ between BALB/c and C57B/6 mice are important in determining pathogenicity in the susceptible BALB/c strain. Amino acid substitutions at residues 741, 806, and 901 make the S2 subunit pathogenic in the nonsusceptible BALB/c strain as well. To elucidate the pathogenesis of autoimmune disease and to devise strategies for preventing or aborting disease, it is important to identify predisposing genes as well as epitopes of the autoantigens that are immunogenic and pathogenic. Further studies of pathogenic epitopes will help define the processing and presentation of this autoantigen to the immune system. The demonstration that allelic differences in an autoantigen contribute to differential susceptibility to autoimmune disease in various strains of mice provides new opportunities for investigating the genetic predisposition to many autoimmune diseases.

C57B/6 myosin. There is a relatively large focus of subepicardial left ventricular free wall myocarditis with several necrotic myocytes (*arrows*) and associated mononuclear inflammatory cells. Hematoxylin-eosin, ×320. (*Bottom*) C57B/6 mouse immunized with the genetically engineered D4 fragment of rat Myhcα (see text). There is a small focus of left ventricular free wall myocarditis with inflammatory cells and at least one necrotic or degenerating myocyte (*arrow*). Hematoxylin-eosin, ×320.

Table II. Induction of Autoimmune Myocarditis by Fragments of Rat Myhcα

Immunogen	Myocarditis BALB/c	Myocarditis C57B/6
D0 (amino acids 1–735)	0/5	
D4 (amino acids 1–1032)	5/5	3/5

BALB/c or C57B/6 mice were immunized with genetically engineered fragments of rat Myhcα D0 or D4, emulsified in CFA, and boosted with the same 7 d later. The heart was removed from each mouse 3 wk later and fixed in formalin. Hearts were sectioned and stained with hematoxylin-eosin.

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