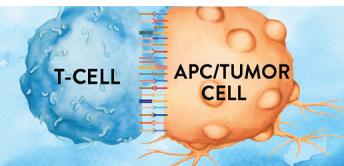


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Mimicry and Antibody-Mediated Cell Signaling in Autoimmune Myocarditis¹

Ya Li,*[†] Janet S. Heuser,* Luke C. Cunningham,* Stanley D. Kosanke,* and Madeleine W. Cunningham^{2*}

The mechanisms by which autoantibodies against cardiac myosin (CM) may lead to heart dysfunction is unknown. We show that autoantibodies to CM in anti-CM sera and mAbs derived from experimental autoimmune myocarditis targeted the heart cell surface and induced Ab-mediated cAMP-dependent protein kinase A activity. Ab-mediated cell signaling of protein kinase A was blocked by CM, anti-IgG, or by specific inhibitors of the β -adrenergic receptor (β -AR) pathway. mAbs confirmed mimicry between CM and the β -AR. Passive transfer of purified Ab (IgG) from CM-immunized rats resulted in IgG deposition and apoptosis in the heart, leading to a cardiomyopathic heart disease phenotype in recipients. Our novel findings link anti-CM Ab with the β -AR and subsequent Ab-mediated cell signaling in the heart. *The Journal of Immunology*, 2006, 177: 8234–8240.

Immunity leading to heart disease is not well understood due to the heterogeneity of immune-mediated heart diseases as well as the lack of knowledge about specific immune mechanisms in the heart. Myocarditis is an inflammatory heart disease that can result in dilated cardiomyopathy (DCM)³ and heart failure (1–4). Although myocarditis may be initiated by viral infection, it is also accompanied by exposure of myocarditic Ags, such as cardiac myosin (CM) (5–8). It has been demonstrated that in myocarditis patients the prevalence of Abs against CM are elevated and associated with the deterioration of cardiac function in patients with chronic myocarditis and cardiomyopathy (9–13). Although autoantibodies against CM may be present in patients with cardiomyopathy and experimental animals with myocarditis, the mechanism of pathogenesis and significance of the anti-CM autoantibodies is unknown.

The clinical manifestations in myocarditis and subsequent cardiomyopathy suggest that the disease may be mediated by both cellular and humoral immune responses directed against CM. Numerous studies have shown that cellular immunity is an important pathogenic determinant in autoimmune myocarditis (14–17). However, immunoadsorption of circulating autoantibodies has been shown to improve the cardiac function of patients with DCM (18). Occurrence of cardiomyopathy in the absence of a T cell

infiltrate would implicate the potential role of Ab in the pathogenesis of disease.

In animal models of myocarditis, CM has emerged as the dominant autoantigen in genetically susceptible animals (19–22). Although the induction of myocarditis by CD4⁺ T cells is well established, very little is known about how Abs may affect the heart. Transfer of anti-CM mAbs led to myocarditis in certain strains of mice (23–25), and the induction of circulating anti-CM autoantibody and IgG deposition in the myocardium was shown to be concomitant with the onset of myocarditis (23, 26). It has been unclear how Abs against an intracellular protein such as CM can affect the heart. Deposition of anti-myosin Ab occurs in damaged tissue where myosin is exposed, but our hypothesis is that the anti-CM Abs recognize molecules expressed on the surface of cardiomyocytes and directly affect the heart. In an effort to understand the potential role and mechanism of autoantibodies against CM in the heart, we investigated whether autoantibody against CM can directly target cardiac myocytes and have a direct role in pathogenesis of heart disease.

Our study shows that anti-CM Abs induced by immunization with CM or its pathogenic peptide S2-16 target the β -adrenergic receptor (β -AR) on the heart cell surface and specifically induce cAMP-dependent protein kinase A (PKA) activity in heart cells. It is well known that binding of the β -AR triggers the major signaling pathway controlling heart rate and contractility (27). Activation of PKA through β -AR induces apoptosis during development of heart failure (28, 29). The effect of anti-CM Ab on the β -AR suggests that CM Abs can modulate myocardial function and severity of cardiomyopathy and heart failure. Recent studies have shown that Abs generated to peptides of the β -AR induce cardiac dysfunction and cardiomyopathy (30). To further support our conclusions, we found that anti-CM Ab-mediated cell signaling of PKA was inhibited by either CM, anti-IgG, or by specific inhibitors of the β -AR pathway. mAbs specific for CM confirmed the cross-reactive mimicry between CM and the β -AR. Finally, passive transfer of purified IgG from CM-immunized rats resulted in IgG deposition, leading to dilated cardiomyopathic changes in the hearts of recipients. Our novel findings implicate anti-CM autoantibody-mediated cell signaling in myocarditis and cardiomyopathy where Abs against CM target the β -AR, alter signal transduction, and lead to cardiomyopathy.

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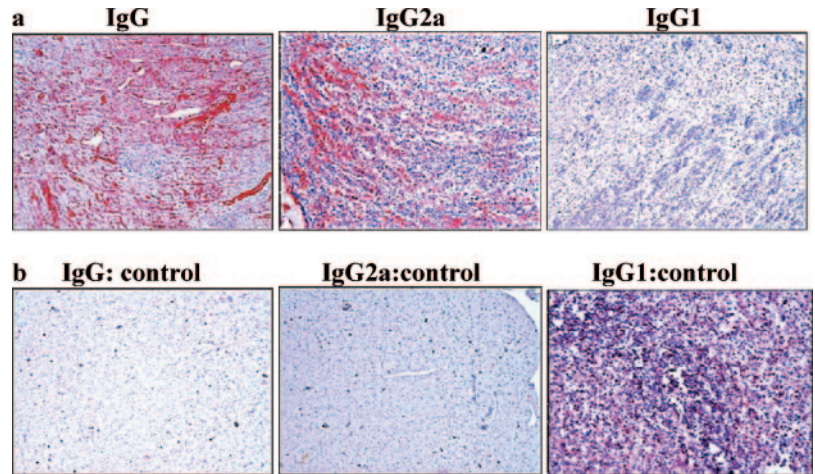
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³ Abbreviations used in this paper: DCM, dilated cardiomyopathy; CM, cardiac myosin; β -AR, β -adrenergic receptor; PKA, protein kinase A; r β 2-AR, recombinant β 2-AR; EAM, experimental autoimmune myocarditis; PLB, phosphorylated phospholamban.

FIGURE 1. In vivo deposition of IgG2a Abs in EAM. Lewis rats were immunized with S2-16 or CM emulsified in CFA (a) or PBS in CFA (b) as a control treatment. Myocardial tissue-bound IgG (left), IgG2a (middle), and IgG1 (right) were detected by immunostaining. IgG1 deposition in spleen (b, right panel) is a positive control for IgG1 immunostaining.



Materials and Methods

Antigens

Purified human CM and S2-16 peptide were prepared as previously described (21). Recombinant human α 2a-, β 1-, and β 2-adrenergic receptors (β 2-AR) were purchased from Sigma-Aldrich.

Heart cell line

Rat primary heart cell line H9c2 was obtained from the American Type Culture Collection. Cells were cultured in IMDM (Invitrogen Life Technologies) containing 10% FCS, 1% penicillin and streptomycin, and 0.1% gentamicin at 37°C in 5% CO₂.

Sera and mAbs from experimental autoimmune myocarditis (EAM)

Sera from CM-, S2h16-, or PBS-immunized rats were collected and stored as described previously (22). Splenic mononuclear cells were isolated from Lewis rats 2 wk after primary immunization, fused with the K6H6/B5 cell line (CRL 1832; American Type Culture Collection), and selected with hypoxanthine, aminopterin, and thymidine as previously described (31). Hybridomas secreting mAbs reactive to CM and β 2-AR were produced by limiting dilution. Concentration and isotype of mAbs were determined by using goat anti-rat Ig specific for different classes and subclasses (Jackson ImmunoResearch Laboratories) in ELISA. All experiments on animals were performed in accordance with relevant institutional (National Institutes of Health and University of Oklahoma Health Sciences Center) guidelines and regulations as set by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee.

Passive transfer of IgG Abs from EAM

IgG Abs were purified from sera by affinity chromatography using rProtein G-Sepharose (Zymed Laboratories). The titers of anti-CM IgG were measured by ELISA and range from 1/6400 to 1/12800. Concentrations of Abs were measured by Bio-Rad protein assay (Bio-Rad) and an ELISA for rat IgG. Purified IgG was i.v. transferred to 6- to 8-wk-old naive Lewis rats. Each rat was given 0.5 mg Ab once a week for 18 wk. One group of age-matched naive rats was used as nontransferred controls. Rats were sacrificed after 18 wk and hearts were removed, weighed, and fixed in 10% buffered Formalin. Paraffin sections were prepared from hearts dissected in the atrial-apical axis to produce two halves in which four chambers could be seen. H&E-stained 5- μ m sections were prepared for microscopic histological examination. The morphometric parameters of

each heart were determined by an Olympus BX40 microscope with a micrometer (Olympus).

ELISA and competitive-inhibition ELISA

ELISA was conducted as described elsewhere (22). Inhibitor solution was prepared in PBS and tested at 500 to 4 μ g/ml. Inhibitors were mixed with an equal volume of Ab dilution (1/250 for rat sera, 1/400 for human sera, and 5–10 μ g/ml for mAbs) and incubated at 37°C for one-half hour and overnight at 4°C. Fifty microliters of serum-inhibitor mixtures was added to 96-well microtiter plates coated with 10 μ g/ml CM or β 2-AR and incubated overnight at 4°C. The remainder of the assay was performed as described in the ELISA procedure.

Western immunoblot

Twenty micrograms per milliliter of Ags was separated by SDS-PAGE in 10% gradient slab gels. The gel was overlaid with a sheet of Immobilon-polyvinylidene difluoride transfer membrane (Millipore). The blot was incubated with serum at a 1/100 dilution, mAbs at 5–10 μ g/ml, or Ab specific for Ser¹⁶-phosphorylated phospholamban (PLB; Badrilla) at 1/5000 overnight at 4°C. A Western blotting luminol reagent (Santa Cruz Biotechnology) was used to develop the blots. The immunoreaction was quantified with a scanning photodensitometer.

Immunostaining of tissues and H9c2 cells

Mouse anti-rat IgG (10 μ g/ml), anti-rat IgG1 (1/100), anti-rat IgG2a (1/100), or isotype-control mouse IgG Ab (10 μ g/ml; Sigma-Aldrich) was incubated on deparaffinized tissue sections for 2 h at room temperature. Biotin-conjugated goat anti-mouse IgG Ab or rabbit anti-goat IgG (1/500; Jackson ImmunoResearch Laboratories) was incubated on tissues for 30 min. Alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories) was incubated on tissues at 1 μ g/ml for 30 min at room temperature. Ab binding was detected with Fast Red substrate (BioGenex) against a counterstain of Mayer's hematoxylin (BioGenex). For H9c2 cell staining, 5 \times 10⁴ H9c2 cells were plated to 4-well chamber slides and incubated overnight at 37°C in 5% CO₂. Sera (1/100 dilution) or mAbs (5–10 μ g/ml) were added to cell culture chambers and incubated for 1 h followed by addition of buffered Formalin. Cell surface-bound Abs were detected with specific biotin-conjugated secondary Ab (1/500; Sigma-Aldrich).

For detection of apoptosis in heart tissues, we used anti-active caspase 3 to determine the presence of active caspase 3 in tissues from Lewis rats passively transferred with control and anti-CM or anti-S2-16 IgG. The presence of caspase 3 indicates apoptosis in tissues. To test for the presence

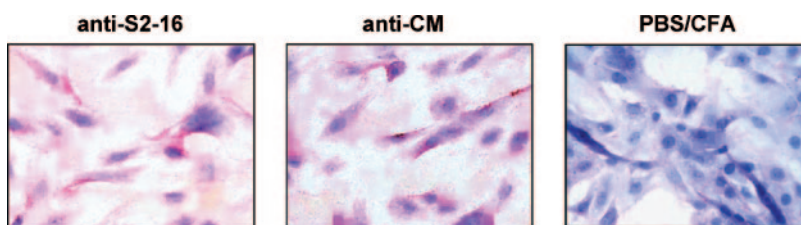
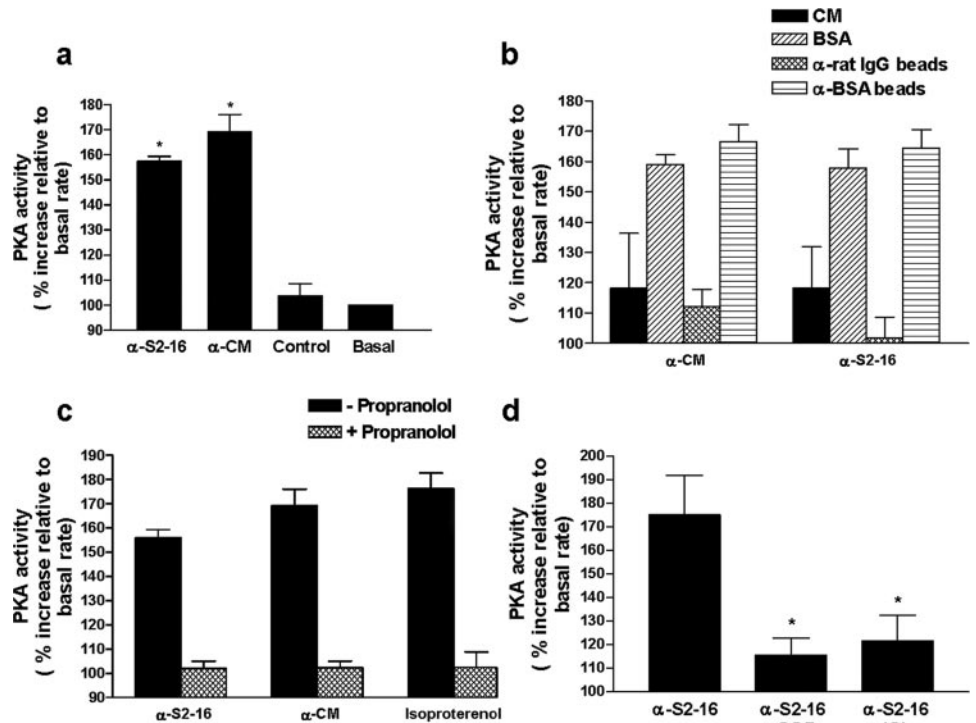


FIGURE 2. Anti-CM and anti-CM peptide S2-16 sera recognized heart cell surface. H9c2 rat heart cells were reacted with anti-S2-16 sera (left), anti-CM sera (middle), or sera from PBS/CFA-immunized control rats (right) and tested for cell surface binding of rat IgG by immunostaining.

FIGURE 3. Anti-CM and anti-S2-16 IgG Abs induce PKA through β -AR. *a*, Anti-S2-16 and anti-CM sera induced PKA activity above the basal rate. *, $p < 0.05$ vs sera from PBS/CFA-immunized control rats. *b*, CM but not BSA abolished anti-CM and anti-S2-16 sera-induced PKA activity. Adsorption with anti-rat IgG also inhibited PKA activity, whereas adsorption with anti-BSA did not. *c*, Anti-S2-16, anti-CM, and β -AR agonist isoproterenol-induced PKA activity was inhibited by β -AR antagonist propranolol. *d*, Anti-S2-16 sera-induced PKA activity was inhibited by β 1 blocker CGP 20712A and β 2 blocker ICI188.551. *, $p < 0.05$ vs anti-S2-16 sera treated only. Values of p were calculated using the unpaired Student's t test.



of caspase 3, Formalin-fixed, paraffin-embedded tissues were deparaffinized 3:1 xylene to heme D for 90 s and rehydrated through a graded ethanol wash. Tissue slides were immersed in a 0.1 M citrate buffer (pH 6.0) and subjected to microwaves three times at 1300 W/s for 90 s with a 60-s break between each microwave session. Slides were washed twice with PBS (pH 7.2). Tissues were then treated for 15 min with crystal violet (2 mg/ml) to block autofluorescence. To detect active caspase 3, an Apo Active 3 kit was used (Cell Technology). Slides were washed three times for 5 min each in PBS plus 0.1% Triton X-100. Tissues were incubated with 100 μ l of rabbit anti-active caspase 3 Ab for 1 h at room temperature. Slides were washed in PBS plus 0.1% Triton X-100 three times for 10 min each. To block tissues, slides were incubated for 20 min in 2% BSA in PBS and then washed and incubated at room temperature for 1 h with 50 μ l of goat anti-rabbit FITC-labeled Ab. Slides were washed three times in PBS, coverslipped, and viewed in an Olympus BX41 microscope equipped with fluorescence.

Protein kinase assays

H9c2 cells (1×10^7) were plated in T75 cell culture flasks overnight. Sera (1/100 dilution) or mAbs (5–10 μ g/ml) were incubated with cells in a final volume of 15 ml of medium for 1 h before reactions were stopped by

ice-cold PBS. Cells were mechanically dislodged from flasks, centrifuged, and solubilized in 0.3 ml of protein extraction buffer before homogenization. Protein kinase activity of H9c2 cells was measured using a SignaTECT PKA assay system (Promega). For inhibition of Ab-induced PKA activity, β blocker propranolol (10 μ M), β 1 blocker CGP 20712A (3 μ M), and β 2 blocker ICI 188.551 (2 μ M) were added. β -AR agonist isoproterenol (10 μ M) was used to induce PKA activity in the heart cells. For competitive inhibition of Ab reactivity, 500 μ g/ml inhibitor CM or BSA was mixed with the serum dilution and incubated as described in the competitive-inhibition ELISA above. The Abs and inhibitor mixtures were added into reaction with H9c2 cells for the PKA assay as described above. Calculation of the units of PKA per microgram of protein was determined and computed in the formula used in the assay kit purchased from Promega. We determined the specific activity of the enzyme in picomoles per minute per microgram for each sample and presented the results as percentages of the basal rate. The PKA activity was then reported as percent above the basal level.

Statistical analysis

Means, SEMs, and unpaired Student's t test and the Mann-Whitney U test were used to analyze data using GraphPad Prism (GraphPad). Groups were

Table I. Monoclonal and serum anti-cm Ab react with β -AR and induce PKA activity

mAb	Reaction with β 2-AR/CM ^a	% Inhibition of mAb by Inhibitor ^b				PKA Activity % Increase ^c (over basal level)
		CM	β 2-AR	α 2a-AR	BSA	
9B6-3E6	0.91/1.42	36.2	23.4	0	1.9	15
3A5-2E10	0.95/1.41	87.5	41.3	0	0	21
2C3-2B6	0.71/1.06	69.7	67.7	3.2	0	30
2C3-2B9	0.93/1.41	89	38	6.3	0	21
Anti-CM sera	0.85/2.1	63.2	82.8	10.7	10	64
Anti-S2-16 sera	0.91/1.82	51.8	83.7	7.9	13.4	56
Sera control ^d	0.15/0.13					4
Isotype control ^d	0.07/0.15					5

^a The reactivities of mAbs with CM and β 2-AR were examined by direct ELISA. Results represent OD reading for each mAb.

^b Competitive-inhibition ELISA of serum (1/250) and mAb reactivity to β 2-AR. Five hundred micrograms per milliliter of inhibitors was used, and percent inhibition was calculated by using OD reading with and without inhibitor. Inhibition assay was not performed on sera and isotype controls.

^c Data represent percentage of increase relative to the basal rate.

^d Sera control, sera from PBS/CFA-immunized rats; isotype control, rat IgM.

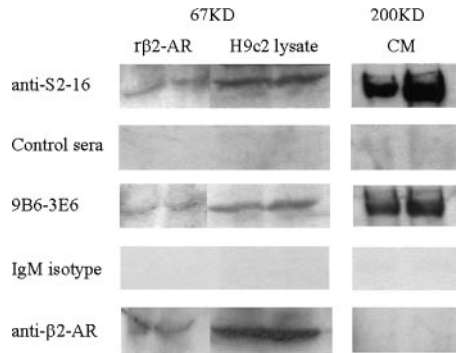


FIGURE 4. Anti-CM mAbs and sera react with β -AR. Western immunoblot of $r\beta$ 2-AR, H9c2 cell lysate, and CM (each Ag was loaded in duplicated lanes) was probed with anti-S2-16 sera, PBS/CFA-immunized control rat sera, mAb 9B6-3E6, isotype control Ab, and anti- β 2-AR Ab. Anti-CM sera and other anti-CM mAbs gave similar results as the anti-S2-16 sera and 9B6-3E6 (data not shown).

considered statistically different if $p \leq 0.05$. The Mann-Whitney U test was used to analyze the data in Table II, whereas the Student's t test was used to analyze the data shown in Fig. 3.

Results

Anti-CM Abs from EAM target the heart cell surface and β -AR

CM and its pathogenic peptide epitope, S2-16, which is derived from the CM S2 rod region, are capable of inducing myocarditis in Lewis rats when injected with CFA (22). As shown in our previous study, IgG Ab against CM was induced in sera of rats after immunization with S2-16 or CM (22). In addition, Abs (IgG and IgG2a) deposited in the myocardium of rats developing myocarditis (Fig. 1*a*), both in regions of cellular infiltration and along intact muscle fibers (Fig. 1). Control rats receiving only adjuvants did not demonstrate any deposition of IgG (Fig. 1*b*). Deposition of Abs in the intact myocardium suggested that anti-CM autoantibodies may directly target the surface of normal cardiomyocytes. To assess cardiomyocyte surface binding of Ab from rats immunized with S2-16 peptide or CM, we reacted anti-S2-16 and anti-CM sera from S2-16 and CM-immunized Lewis rats with rat heart cell line H9c2. Binding of Abs to rat heart cells was fixed and then detected by immunostaining of rat IgG. We found both anti-S2-16 (Fig. 2, *left*) and anti-CM Abs (Fig. 2, *middle*) deposited on the surface of H9c2, which suggested a heart cell surface-specific reaction. PBS/CFA-immunized rat sera were tested as a negative control and showed no staining (Fig. 2, *right*). Sera against control S2 peptide, such as S2-18, from S2-18/CFA-immunized rats was also negative in H9c2 cell staining (data not shown).

Binding of Abs from S2-16- and CM-immunized rats to the heart cell surface suggested that Abs may contribute to tissue injury. In the heart, cAMP-dependent PKA signaling activated by stimulation of the β -AR is the most powerful mechanism to enhance myocardial performance in response to stress or exercise. To determine whether PKA activity was induced by autoantibodies from EAM, we analyzed Ab-mediated activation of PKA in the rat heart cell line H9c2. As shown in Fig. 3*a*, pooled anti-S2-16 and anti-CM sera but not sera from PBS/CFA-immunized control rats increased PKA activity to 50–70% above the basal level. In contrast, we did not detect calcium/calmodulin-dependent protein kinase II activation by anti-S2-16 and anti-CM sera (data not shown).

To confirm that CM plays a role in Ab-induced heart cell signaling, we demonstrated that anti-S2-16 and anti-CM sera-induced PKA activity was inhibited by CM but not BSA adsorption (Fig.

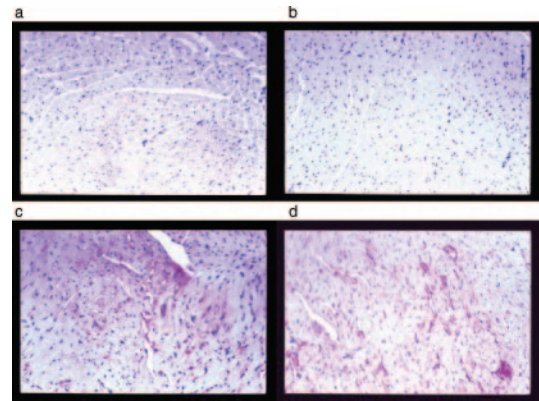


FIGURE 5. Passive transfer of anti-S2-16 sera into naive Lewis rats leads to in vivo IgG deposition in the heart. Control rats receiving serum from PBS/CFA-immunized control rats (*a* and *b*) did not show IgG deposition, whereas rats treated with anti-CM or anti-S2-16 serum (*c* and *d*) demonstrated IgG deposition in the myocardium.

3*b*). The data suggested that CM-reactive Ab was responsible for Ab-induced PKA activity. In addition, the induction of PKA activity was inhibited when IgG was absorbed from anti-S2-16 and anti-CM sera (Fig. 3*b*). Anti-BSA adsorption, as a control, did not affect PKA activity induced by sera (Fig. 3*b*). These results suggested that the induction of PKA activity was mediated by CM-reactive IgG Ab in anti-S2-16 and anti-CM sera.

To demonstrate that Ab-induced heart cell signaling was mediated by the β -AR, propranolol, a β -AR antagonist, effectively inhibited β -AR agonist isoproterenol-induced PKA activity as well as anti-CM or anti-S2-16 sera-induced PKA activity (Fig. 3*c*). Therefore, anti-CM Abs act as an agonist of β -AR. Furthermore, anti-S2-16 sera-induced PKA activation was inhibited by both β 1 blocker CGP and β 2 blocker ICI (Fig. 3*d*), which suggested that both β 1- and β 2-AR were stimulated by anti-CM Abs and contributed to Ab-induced PKA activation. The data are consistent with the fact that stimulation of β 1- and β 2-AR can both increase levels of secondary messenger cAMP and PKA activity and that β 1- and β 2-AR share >50% amino acid sequence identity.

Anti-CM Abs cross-react with β -AR

Because anti-CM Ab-induced PKA activity was inhibited by CM and β -AR antagonists, CM-reactive Abs may recognize the β -AR and function as an agonist. To further assess the potential cross-reactivity of anti-CM Abs, rat hybridomas were established from the fusion of splenic mononuclear cells of CM-immunized rats

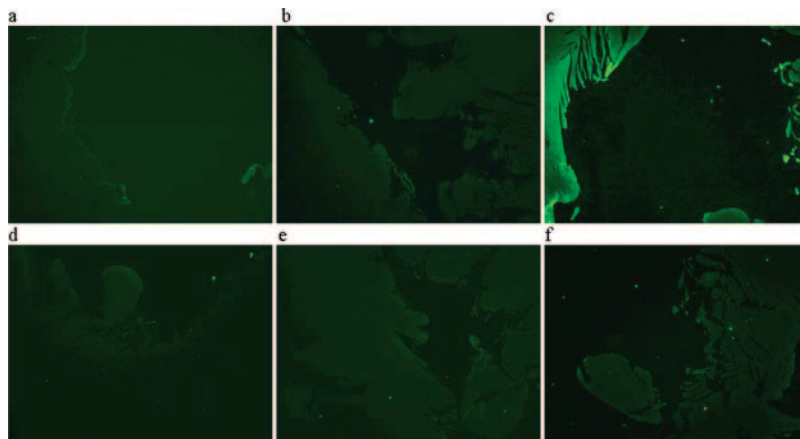
Table II. Morphology and IgG deposition in hearts of rats treated with Ab^a

	Naive ($n = 3$)	Control IgG-Transferred Rats ($n = 3$)	Anti-CM IgG-Transferred Rats ($n = 4$)
HW \times 100/BW	0.29 \pm 0.01	0.28 \pm 0.003	0.31 \pm 0.009
LVCD (mm)	58.7 \pm 1.3**	61 \pm 12.1*	90.8 \pm 3.3
RVCD (mm)	12.7 \pm 1.3	13.7 \pm 2	16.7 \pm 3.5
LVPW (mm)	41.3 \pm 2.9*	36.3 \pm 0.7*	32 \pm 1.4
LVS (mm)	34.3 \pm 0.3*	33 \pm 0.6*	27.2 \pm 1.1
IgG deposition ^b	0*	0.2 \pm 0.1*	1.8 \pm 0.4

^a HW, heart weight; BW, body weight; LVCD, left ventricular cavity diameter; RVCD, right ventricular cavity diameter (magnification, $\times 4$); LVPW, left ventricular posterior wall; LVS, left ventricular septum. Values are mean \pm SEM. *, $p < 0.05$; **, $p < 0.001$ vs the anti-CM IgG-transferred rats group as calculated by the Mann-Whitney U test.

^b The staining reaction was graded from no staining (0+) to maximum.

FIGURE 6. Anti-caspase 3 FITC-stained cardiac sections demonstrate apoptosis in rats treated with anti-CM Ab. *a*, Control tissues from naive Lewis rats did not show binding of the anti-caspase 3. *b*, Rats passively transferred with serum from rats immunized with PBS/CFA also did not show binding of the anti-caspase 3. *c*, However, rats treated with anti-CM IgG demonstrated binding of anti-caspase 3, suggesting the presence of apoptosis. Caspase 3 was imaged using rabbit anti-caspase 3 and anti-rabbit FITC-conjugated Ab to detect anti-caspase 3 binding and apoptosis. Control tissues stained with both Abs are shown in *a* and *b*, and matching control tissues (*d-f*) were stained with only anti-rabbit FITC-conjugated Ab.



with the K6H6/B5 cell line. Thirteen hybridomas were selected, cloned, and expanded for production of mAbs reacting with CM and $r\beta 2$ -AR. Table I shows four representative mAbs (9B6-3E6, 3A5-2E10, 2C3-2B6, and 2C3-2B9) which reacted strongly with both CM and $r\beta 2$ -AR. Anti-CM and anti-S2-16 sera also reacted with both CM and $r\beta 2$ -AR, whereas sera from PBS/CFA-immunized rats (sera control) and isotype control Ab rat IgM did not. mAbs were not reactive to control Ags such as tropomyosin and laminin (data not shown). When Ab-induced PKA activity in H9c2 heart cells was tested, mAbs induced PKA activity 15–30% above the basal level. Anti-CM and anti-S2-16 sera increased PKA activity 50–70% above basal level, whereas control sera and rat IgM isotype control did not induce PKA activity (Table I). The weaker PKA reactivity induced by the cross-reactive mAbs was not unexpected compared with the stronger sera.

The reaction of anti-CM mAbs and sera from rats immunized with CM peptide S2-16 or CM with the β -AR was confirmed by Western immunoblot. Anti-S2-16 sera, anti-CM sera (data not shown), and CM-reactive mAbs (9B6-3E6 as a selected sample) reacted with both the H chain of CM (200 kDa) and $r\beta 2$ -AR (67 kDa), and a protein in H9c2 cell lysates that had the same molecular mass as the $\beta 2$ -AR (Fig. 4). Control sera from PBS/CFA-immunized rats and an IgM isotype control for mAbs did not have such reactivity (Fig. 4). As a positive control, purified anti- $\beta 2$ -AR Ab recognized both $r\beta 2$ -AR and the 67-kDa protein in H9c2 cells but not CM in Western blots (Fig. 4).

To further confirm the reactivity of the anti-CM Abs with the β -AR, we performed a competitive-inhibition ELISA. CM, $r\beta 2$ -AR, recombinant α_{2a} -adrenergic receptor, and BSA were tested to determine their capacity to inhibit the reaction of anti-CM Abs with $r\beta 2$ -AR. As shown in Table I, the reaction of anti-CM mAbs or sera with $r\beta 2$ -AR was markedly inhibited when $r\beta 2$ -AR or CM was used as an inhibitor, whereas neither α -AR nor BSA inhibited the sera or mAb binding to β -AR (Table I). In competitive inhibition ELISA, a dose-dependent inhibition was observed with mAbs (500 to 4 μ g/ml) and sera (500 to 15.6 μ g/ml; data not shown).

Taken together, these results suggested that the immunological cross-reactivity between CM and β -AR may be a potential mechanism leading to recognition of the β -AR and subsequent Ab-induced PKA activity in cardiomyocytes.

In vivo effects of cross-reactive anti-CM Ab

We also examined the possible effects of CM and β -AR cross-reactive Ab in vivo by passive transfer. As shown in Fig. 5, *a* and *b*, repeated i.v. injection for 2 wk of purified high-titer anti-CM/ β -AR IgG Ab (6.75 μ g total) from CM- or S2-16-immunized rats

resulted in Ab deposition in the myocardium as shown in Fig. 5, *c* and *d*. In contrast, i.v. injection of control anti-PBS IgG did not lead to IgG deposition (Fig. 5, *a* and *b*). Therefore, when we transferred 0.5 mg purified IgG once every week for 18 wk, Ab deposition was evident in hearts of the recipients (Table II and Fig. 5, *c* and *d*). In anti-CM IgG-transferred rats, the left ventricular cavity diameter was significantly increased and was accompanied by a decrease in thickness of the septum and posterior wall, compared with naive rats receiving no IgG and rats receiving control PBS/CFA-immunized rat IgG. The ratio of heart weight to body weight also tended to be higher in the CM-IgG-transferred group, but it failed to reach statistical significance (Table II). Histological examination did not reveal any cellular infiltration or necrosis in the anti-CM-IgG-transferred rats as well as control recipients. These results suggested that cross-reactive anti-CM autoantibodies do not directly cause histological inflammatory effects but may induce a cell-signaling cascade and myocardial changes leading to heart dysfunction and cardiomyopathy. In further studies, we found apoptosis in the hearts of animals receiving anti-CM IgG as shown in Fig. 6. Rats treated with anti-CM IgG (Fig. 6*c*) demonstrated binding of anti-caspase 3 and apoptosis. Control tissues from animals receiving IgG from adjuvant-immunized rats did not show the presence of caspase 3 and were not apoptotic (Fig. 6*b*). Further control rats, including hearts from naive animals (Fig. 6*a*), also did not show any apoptosis. Control tissues stained only with the anti-rabbit IgG FITC-conjugated Ab were also negative as shown in Fig. 6, *d-f*. Therefore, the data suggest that passively transferred anti-CM IgG may lead to apoptosis and thinning of the myocardium as shown in Table II.

Discussion

The role of humoral immunity in autoimmune heart disease has not been established. Although immunization with CM leads to myocarditis and cellular infiltration in the heart, the mechanisms by which Ab may affect the heart have not been determined. Our novel findings show that anti-CM autoantibodies in experimental autoimmune myocarditis cross-react with the β -AR and induce signal transduction in heart cells. Our data support the hypothesis that anti-CM autoantibody in myocarditis may have pathophysiological effects on heart cells due to Ab cross-reactivity. Therefore, molecular mimicry and cross-reactivity of cardiac autoantibodies may be an important mechanism by which Ab may contribute to heart disease.

Circulating cardiac autoantibodies to CM and other heart-specific autoantigens are markers of autoimmunity in human myocarditis (11, 12, 32–36). In mice, deposition of CM-reactive Abs in the myocardium was detected after immunization with CM (26). In

this study, we observed Ab deposition in the hearts of Lewis rats immunized with CM and the pathogenic CM peptide S2-16. When the Ab subclass deposition pattern was defined, IgG and the IgG2a subclass but not IgG1 Abs were identified in the heart tissues in Lewis rat EAM (data not shown). This result may reflect a Th1 inflammatory and pathogenic Ab response. Ab recognition of heart tissue may also be mediated by exposed CM after myocardial injury. However, cardiac Ab deposition was observed in both inflammatory lesions of heart as well as in normal heart tissues. Although Ab binding to heart tissue might be explained by an aberrant expression of CM on heart cells (23), alternatively, the binding of CM-reactive Abs to heart tissue may be mediated by other heart cell surface molecules. In this study, we demonstrated heart cell surface binding by anti-CM Abs that recognized the β -AR on heart cells. Our report suggests that the potential pathogenic effects of anti-CM autoantibodies are related to heart cell surface recognition.

In the heart, one of the most important signaling receptors is the β -AR (27). PKA activation induced by β -AR stimulation is an important mechanism to enhance myocardial performance in response to stress or exercise (27). In this study, Ab-mediated activation of PKA was identified in a rat heart cell line and was shown to be mediated by both β 1- and β 2-AR. These results are consistent with previous reports suggesting that sera from myocarditis and cardiomyopathy patients contain agonistic autoantibodies that bind to β -AR (34, 37). Adrenergic receptors, especially β 1 subtype, not only function to generate second messengers to control the heart contractility, but also to regulate other cardiac functions, myocyte growth, and programmed cell death. Similar mechanisms may be responsible for signal transduction by cardiac autoantibodies in human and animal myocarditis.

Heart cell signaling induced by anti-CM autoantibodies suggested that Abs deposited in myocarditic hearts may have pathogenic effects physiologically rather than histologically. This is demonstrated by our Ab passive transfer experiment. Although we did not examine the physiological function of rat hearts after Ab transfer, morphometric analysis showed that hearts from anti-CM IgG-transferred rats had enlarged left ventricular chambers and thinner septum and posterior walls than those of control-transferred rats. An enlarged left ventricular chamber and thinning septum and wall are all features of DCM (3). These results are consistent with a recent report by Jahns et al. (30), where immunization against the β 1-AR second extracellular loop peptide resulted in anti- β -AR Ab, which induced cardiomyopathy by both immunization and passive transfer.

In this study, anti-CM autoantibody-induced PKA activity was shown to be inhibited by either CM or β -AR antagonists. Our observation suggested a potential Ab cross-reaction between CM and β -AR. To further confirm the cross-reaction between CM and β -AR, we derived hybridomas from rats immunized with CM. mAbs and sera from CM- or S2-16-immunized rats reacted strongly to the 200-kDa H chain of CM and the 67-kDa β 2-AR in both ELISA and in Western blot analysis. In addition, β 2-AR was shown to be a potent inhibitor of anti-CM Ab reactivity with CM and vice versa. β 2-AR was used in this study because of its availability. Both β 1- and β 2-AR have the same molecular mass and have >50% amino acid sequence identity. Our data indicated mimicry between two cardiac Ags, CM and β -AR, which lead to a humoral mechanism for induction of PKA activity in heart cells. This is the first time that the β -AR has been linked with immune responses against CM, the dominant autoantigen in autoimmune myocarditis. Mimicry between CM and β -AR may not be apparent, although both of these two proteins have α helical structure. CM has coiled-coil α helical conformation, whereas β -AR is a

seven-transmembrane-spanning receptor. Protein sequence alignment between CM and β 1-AR showed a 30% identity and a 56% homology in a 57 aa overlap (CM, 98-154; β 1-AR, 300-348), and alignment between CM and β 2-AR showed a 25% identity and a 57% homology in an 85 aa overlap (CM, 543-624; β 2-AR, 131-213). Most sequence homology alignment was within surface-exposed regions of the β -AR. The defined S2-16 epitope of CM that induced Ab cross-reactive with β -AR demonstrated a 44% identity and a 78% homology with β 1-AR in a 9 aa overlap (S2-16, 5-13; β 1-AR, 48-56) and a 34% identity and a 67% homology with β 2-AR in a 12 aa overlap (S2-16, 12-23; β 2-AR, 396-407). However, it can be difficult to define Ab-reactive sites in the molecules because the Abs most likely react with conformational epitopes. It has been shown that a single Ab is capable of adopting different binding site conformation and can bind unrelated Ags (38, 39). In mimicry and cross-reactivity by an Ab, conformational diversity increases the Ab repertoire but also reduces Ab specificity and avidity.

We believe that the major contribution of this study is the demonstration that anti-CM Abs cross-react with and signal the β -AR and therefore might lead to cardiomyopathy in animals or humans. Chronic or excessive stimulation of the β -AR has been shown to promote cardiomyocyte apoptosis through PKA (28, 29). Our novel data demonstrate how anti-CM Abs might alter cardiac function. The data in this report provide an important mechanistic advance previously unknown about anti-myosin Ab responses. Although we were unable to provide data showing impaired cardiac function in our animals, our data show that hearts treated with passively transferred Ab appear to undergo apoptosis. Further preliminary studies suggested that passively transferred anti-CM or anti-S2-16 IgG for 18 wk caused increased PLB at Ser¹⁶ in the myocardium, a result of PKA activation. Continued signaling of PKA may lead to apoptosis in the heart, over time leading to a decrease in myocardial mass. These data could explain the development of a cardiomyopathic heart disease phenotype in animals receiving anti-CM IgG.

In summary, this study links anti-CM Ab to the heart cell surface β -AR and the induction of PKA activity. Molecular mimicry and Ab cross-reactivity appear to serve as humoral mechanisms that may play an important role in the pathogenesis of myocarditis and cardiomyopathy. In addition, cross-reactive autoantibody-mediated cardiomyocyte signaling may contribute to the confounding and often histologically silent clinical manifestations in myocarditis. The finding that anti-CM Abs can modulate cardiomyocyte physiology provides a more complete understanding of the immunopathogenesis of autoimmune myocarditis and DCM.

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