Relationship of Myocardial Remodeling to the Genesis of Serum Autoantibodies to Cardiac Beta1-Adrenoceptors and Muscarinic Type 2 Acetylcholine Receptors in Rats

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OBJECTIVES We sought to investigate the mechanism responsible for the occurrence of anticardiac receptor autoantibodies.

BACKGROUND Increasing evidence suggests the involvement of autoimmune mechanisms in the pathogenesis of a number of cardiovascular diseases. Among them, the biologic, functional and pathogenic properties of anticardiac receptor antibodies have been extensively investigated. However, the mechanism responsible for the occurrence of anticardiac receptor autoantibodies remains poorly understood.

METHODS Two rat models (aortic banding [AB] and adriamycin [ADR] groups) were constructed. Determination of cardiac function and morphology and T-lymphocyte subtypes, enzyme-linked immunosorbent assay and cardiomyocyte cultures were performed.

RESULTS It was shown, in the AB and ADR groups, that the frequency and titer of autoantibodies to beta1 and muscarinic type 2 receptors were increased when myocardial remodeling occurred, as evidenced by significant cardiac morphologic changes, deposition of collagen and obvious functional impairment. This suggests that cardiac remodeling itself, in two disparate models of heart failure and cardiomyopathy, was able to trigger the genesis of anticardiac receptor autoantibodies. These autoantibodies have biologic effects similar to those seen in human autoantibodies. They have also shown a characteristic self-growth, as well as a time-course decline, suggesting that a negative finding of anticardiac receptor autoantibodies in sera of patients with heart disease does not necessarily imply that there is no autoimmune reaction involved in the pathogenesis.

CONCLUSIONS Our results demonstrated that myocardial damage was able to trigger the occurrence of an autoimmune reaction, resulting in the genesis of anticardiac receptor autoantibodies with properties similar to those seen in patients with idiopathic dilated cardiomyopathy. (J Am Coll Cardiol 2002;39:1866–73) © 2002 by the American College of Cardiology Foundation

In recent years, autoantibodies to cardiac beta1-adrenoceptors and muscarinic type 2 (M2) acetylcholine receptors have been found in the sera of patients with dilated cardiomyopathy (DCM) (1–3). Matsui et al. (3) have confirmed the presence of autoantibodies against a series of G-protein–coupled cardiovascular receptors in patients with DCM and found that circulating antireceptor autoantibodies in these patients were mainly confined to two functionally predominant cardiac receptors—beta1-adrenoceptors and M2 receptors—rather than other G-protein–coupled receptors. Further studies have shown that these antireceptor autoantibodies are functionally active (1–5), suggesting the involvement of these autoantibodies in the pathogenesis of DCM. However, apart from DCM, autoantibodies to cardiac beta1-adrenoceptors and M2 receptors have also been found in Chagas’ heart disease, rheumatic heart disease, hypertensive cardiomyopathy and myocardial infarction associated with heart failure (6–10). These findings suggest that the anticardiac receptor autoantibodies are not restricted to patients with DCM, but can also be found in some other cardiac diseases associated with obvious cardiac structural and functional changes. Little information, however, is available regarding the mechanism for the genesis of autoantibodies to cardiac receptors in the course heart diseases. A clarification of the relationship between the myocardial pathologic state and the genesis of antireceptor autoantibodies may deepen our understanding of the role of the autoimmune mechanism in the pathogenesis of heart diseases.

We have extended the previous studies in this field to answer the following questions. 1) How is the myocardial remodeling process during the development of heart failure related to the genesis of autoantibodies to cardiac beta1-adrenoceptors and M2 receptors? To answer this question, we first set up two experimental animal models that had undergone myocardial remodeling, and then we determined their serum antireceptor autoantibodies before and after the experiment. 2) What are the biologic and pharmacologic properties of the autoantibodies generated, if any, in relation to the myocardial remodeling process? To answer this

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question, we determined the functional role of the immunoreactive autoantibodies to cardiac receptors generated in such a way.

**METHODS**

**Animal models.** Healthy 12-week-old Wistar rats of either gender, with normal blood pressure and heart rate, weighing 200 to 240 g, were selected for this study. Animals were classified randomly into two experimental groups, with respective control groups (n = 24 in each group), as follows: 1) the aortic banding (AB) group, in which the abdominal aorta was banded according to the methods of Doering et al. (11). In the sham-operated control group, the animals were treated with the same surgical procedure, except for AB. 2) The adriamycin (ADR) (doxorubicin) group, in which ADR at 1.0 mg/kg body weight per week was administered through the sublingual vein (12). In its corresponding control group, a same volume of normal saline replaced adriamycin. Throughout the experiment, 1 ml of blood sample was taken from all groups through the caudal vein before and 1, 2, 4, 6, 8, 10, 12 and 14 weeks after treatment. The sera were separated and stored at −70°C until use. At 2, 8 and 14 weeks after treatment, four animals each time were anesthetized with 40 mg/kg sodium pentobarbital intraperitoneally, and left ventricular (LV) function was measured invasively. The heart was then excised and frozen in liquid nitrogen and stored at −70°C until use.

**Measurement of in vivo cardiac function.** After anesthesia, a cannula was inserted into the LV to measure the following primary and derived variables: heart rate, LV systolic pressure, LV end-diastolic pressure and the maximal rate of rise and/or decline in left ventricular pressure (±dP/dt max). After completion of the measurements, the blotted weight of the whole heart was weighed. A mid-LV section (about 2 mm in thickness) perpendicular to the major axis of the heart was made and fixed in 10% formalin.

**Cardiac anatomic measurements.** The wall thickness, cavity dimension, cavity area and total ventricular wall area were measured at the mid-sections of each heart after sectioning and staining with hematoxylin-eosin. The measurements were made using a computerized image analysis system (MIANS-300, Sichuan, China). The total ventricular wall area included the ventricular area, interventricular septum, trabeculae carnae and papillary muscles attached to the wall.

**Inspection of cardiac structure.** The cardiac structure was examined by routine light microscopy. The myocardial ultrastructure was inspected by transmission electron microscopy. For this purpose, small pieces of heart tissue were fixed with 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 2 h at 4°C. They were washed with phosphate buffer and then post-fixed with 1% osmium tetroxide for 2 h at 4°C. After dehydration with a graded series of dehydrating acetone, the specimens were infiltrated with epoxy 618. Ultra-thin sections (50 nmol/l in thickness) were cut using the LKB ultra-microtome IV after polymerization at 60°C, stained with a solution of uranyl acetate and lead citrate, and observed under a 100-CX transmission electron microscope.

**Myocardial collagen staining and image processing.** The histologic section of ventricular tissue was stained with van Gieson’s solution of trinitrophenol and acid fuchsin (a kind of collagen staining) for microscopic inspection and image processing. Six microscopic fields per section were sampled. The percentage of collagen tissue per field was calculated, and the final average was adopted as the percent collagen volume fraction (CVF).

**Enzyme-linked immunosorbent assay (ELISA).** Peptides corresponding to the sequence of the second extracellular loops of human beta1-adrenoceptors and M2 receptors were synthesized, and ELISA was performed as previously described (2–4).

**Determination of CD3+ , CD4+ and CD8+ T cells.** For measurement of CD3+, CD4+ and CD8+ T lymphocytes in the rat’s peripheral blood, preparation of samples and determination by flow cytometry (FACScan, Becton Dickinson and Co., Mountain View, California), as well as analysis with the Cellquest Software on an Apple Power Macintosh, were carried out as described by Lyons and Parish (13). The following monoclonal antibodies were used for combining T lymphocyte: fluorescein isothiocyanate (FITC)-labeled anti-rat CD3, FITC-labeled rat CD4 and phycoerythrin-labeled rat CD8 (Pharmingen Co., San Di ego, California).

**Binding characteristics of beta1-adrenoceptors and M2 receptors.** Tissue preparation was done using the methods reported by Vandermolen et al. (14). Normal sections of rat cardiac tissue were used as the source of both receptors for radioligand binding. Ventricular tissues were trimmed to a dimension of 5 × 5 mm and embedded in OCT (Miles). The tissue sections were consecutively cut to 16 µmol/l in thickness on a cryostat (−18°C) microtome (Ryocut 1800). One of five consecutive sections, which formed a set, was used to measure tissue protein by the routine method. Iodine-125-pindolol and ³H-quinuclidinyl benzilate were
used as radioligands for the beta_1-adrenoceptor and M_2 receptor, respectively. The binding assay was carried out by binding the specific ligands directly to the frozen slide tissues. Saturation-binding isotherms were obtained by incubating the sections for 1 h with varying concentrations of either iodine-125-pindolol or H-quinuclidinyl benzylate. The saturation-binding variables of the maximal number of binding sites (B_max) and the dissociation constant (K_d) were determined using the Prism 2.01 program. The activities of the positive sera against beta_1-adrenoceptors and M_2 receptors were observed after pre-incubation of these sera with heart tissue sections for 60 min at 37°C, before binding studies were performed.

Culture of neonatal, beating cardiomyocytes. The hearts were removed aseptically from one- to two-day-old Wistar rats and cultured as previously described (5). The number of beats of a selected isolated myocardial cell or a cluster of synchronously contracting cells in each of 10 fields was counted for 15 s each time. The rat serum, synthetic antigen peptide and corresponding receptor agonists were added, and the cells were observed 5 min after each addition. This procedure was repeated twice in different cultures to yield results representing a total of 30 cells or cell clusters. The basal heart rate was 140 ± 20 beats/min.

Statistical analysis. Results are expressed as the mean value ± SD. The average of the antibody titer was expressed as the geometric mean value. The normality of the geometric mean value of antibody titer was tested using the geometric mean value. The normality of the values was tested using the Shapiro-Wilk test (applicable for a sample size of 50 to 1,000). A comparison among groups was done using analysis of variance with SPSS version 10.0 software (SPSS Inc., Chicago, Illinois). Values of p < 0.05 were considered as significant.

### Table 1. Change in Heart/Body Weight Ratio (mg/g) in Wistar Rats Treated With AB or ADR After 2, 8 and 14 Weeks (n = 8)

<table>
<thead>
<tr>
<th>Week</th>
<th>Sham</th>
<th>Operation</th>
<th>AB</th>
<th>Saline</th>
<th>ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.15 ± 0.09</td>
<td>3.35 ± 0.15*</td>
<td>2.18 ± 0.10</td>
<td>2.25 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.25 ± 0.11</td>
<td>4.08 ± 0.23*</td>
<td>2.29 ± 0.12</td>
<td>4.04 ± 0.20‡</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2.22 ± 0.12</td>
<td>3.73 ± 0.30*</td>
<td>2.23 ± 0.15</td>
<td>3.74 ± 0.18§</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05, †p < 0.01 versus sham-operated control group. ‡p < 0.05 versus saline control group. Data are presented as the mean value ± SD.

AB = aortic banding; ADR = adriamycin.

### RESULTS

Changes in heart weight to body weight ratio. Two weeks after treatment, the heart weight to body weight ratio increased significantly in the AB group, whereas no obvious changes were observed in the ADR group. Eight weeks after treatment, however, in both groups, the heart weight to body weight ratio was considerably increased, as compared with that of each control group. Fourteen weeks after treatment, the heart weight to body weight ratio in both groups tended to decrease, as compared with that at eight weeks after treatment, but it was still significantly different from that in each control group by term, as shown in Table 1. In both experimental animal models, the gross pathologic changes characteristic of congestive heart failure, including excessive hydrocardia and ascites, an enlarged, pale kidney and cardamom liver with central tubule necrosis, were observed at 14 weeks after treatment.

Cardiac function. As shown in Table 2, there were no significant changes in LV function for both groups after two weeks of treatment. Eight weeks after treatment, however, the LV systolic and diastolic functions, expressed by +dP/dt_max and −dP/dt_max, as well as other variables (data not shown), were remarkably decreased in both groups, compared with the control group. Fourteen weeks after treatment, the variables of systolic function showed no further decreasing tendency, whereas the variables of diastolic function, such as −dP/dt_max, were even more decreased than those at the preceding time point.

Cardiac anatomic measurements. Longitudinal sections of the hearts from the ADR group, at the end of the experiment, showed dilation of both the left and right ventricles, with wall thickening. The hearts from the AB group, upon death within 12 weeks of the initial operation, were conspicuously large in size and showed markedly thickened LVs, whereas the hearts from the control group showed a normal chamber size and wall thickness (Table 3).

Light and electron microscopic findings. In both groups, the morphologic examinations of the hearts, by light and electron microscopy, displayed a significant remodeling process, as shown by myocyte hypertrophy, elongation, multifocal degeneration and necrosis, focal myofibrillar lysis, hypercontraction banding of myofibrils, mitochondrial vacuolation and condensation. No histologic changes were found in the hearts of the two control groups.

### Table 2. Left Ventricular Function, Expressed as ±dP/dt_max (kPa/s), in Each Group at Different Times (n = 6)

<table>
<thead>
<tr>
<th>Post Treatment Week</th>
<th>Control Group*</th>
<th>AB Group</th>
<th>ADR Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+dP/dt_max</td>
<td>−dP/dt_max</td>
<td>+dP/dt_max</td>
</tr>
<tr>
<td>2</td>
<td>359.8 ± 27.3</td>
<td>239.8 ± 10.1</td>
<td>317.2 ± 30.9</td>
</tr>
<tr>
<td>8</td>
<td>367.6 ± 29.8</td>
<td>228.1 ± 12.4</td>
<td>202.4 ± 39.0†</td>
</tr>
<tr>
<td>14</td>
<td>340.8 ± 27.2</td>
<td>223.7 ± 12.2</td>
<td>213.0 ± 40.3</td>
</tr>
</tbody>
</table>

*Including both the sham-operated group and saline group. †p < 0.05, ‡p < 0.01 versus the corresponding control group. Data are presented as the mean value ± SD.

Abbreviations as in Table 1.

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Myocardial collagen network remodeling. In both experimental groups, the LV CVF percentages were all considerably higher than those in the corresponding control groups after 8 and 14 weeks, as shown in Table 4. Microscopically, a lot of collagen deposition could be seen around the myocardial small vessels in both experimental models at two weeks after treatment. Deposition of collagen became more prominent and was accompanied by increased tissue space and collagenous fibers at eight weeks after treatment. Furthermore, a thickened collagen compartment appeared around the small vessels, and a more extensive and severe deposition of collagen was observed in between the myocardial fibers after 14 weeks of treatment.

Changes in the T lymphocyte subpopulation. The increase in the CD4\(^+\)/CD8\(^+\) ratio denotes the enhancement of positive immunoregulatory function and, hence, promotes the production of antibody. As shown in Table 5, the ratios of CD4\(^+\)/CD8\(^+\) increased remarkably after 2 and 8 weeks in the AB, as compared with pretreatment, but were normalized after 14 weeks. In the ADR group, there were changes similar to those in the AB group at 2 and 8 weeks, but the ratio of CD4\(^+\)/CD8\(^+\) still remained at a high level at 14 weeks after treatment.

Detection of serum autoantibodies to beta\(_1\)-adrenoceptor and M\(_2\) receptor before and after treatment. In both experimental groups, the frequency of occurrence and titer of autoantibodies to beta\(_1\)-adrenoceptor and M\(_2\) receptors in the sera before treatment were at a very low level. However, these were remarkably increased in both experimental groups after four weeks of treatment (Fig. 1). Moreover, the serum levels varied as a function of time (Fig. 2).

Effects of autoantibodies on ligand binding to the corresponding receptor. In both the AB and ADR groups with positive sera for the beta\(_1\)-adrenoceptor, the normal B\(_{\text{max}}\) value of 35.1 ± 8.2 fmol/mg was decreased to 17.1 ± 9.1 fmol/mg and 18.2 ± 7.4 fmol/mg (both p < 0.01), respectively, although the K\(_d\) was not significantly changed. Similarly, both groups with positive sera for the M\(_3\) receptor had a decrease in B\(_{\text{max}}\) from the normal value of 71.3 ± 10.5 fmol/mg to 36.4 ± 9.8 fmol/mg and 34.2 ± 7.3 fmol/mg (both p < 0.01), respectively, and an increase in the K\(_d\) from the normal value of 3.80 ± 0.37 nmol to 6.70 ± 0.22 nmol and 7.10 ± 0.20 nmol (both p < 0.01), respectively. The effects of positive sera (autoantibodies) were dose-dependent, reaching a maximal decrease in B\(_{\text{max}}\) of ~50%. The inhibitory effects of autoantibodies in both experimental groups were abolished by incubating the positive sera for both receptors with anti-rat immunoglobulin G monoclonal antibody.

Chronotropic effect on cultured cardiomyocytes. The positive sera for the beta\(_1\)-adrenoceptor from both experimental groups were able to increase the heart beat frequency, in the same way as the effect of the beta\(_1\)-adrenoceptor agonist isoprenaline. This effect of the anti-beta\(_1\)-adrenoceptor autoantibody in positive sera was abolished by the addition of the beta\(_1\)-adrenoceptor antagonist propranolol, or by pre-incubating the sera with the corresponding antigenic peptide. In contrast, the positive sera for the M\(_2\) receptor from both experimental groups were able to decrease the heart beat frequency, in the same way as the effect of the muscarinic agonist carbachol. Likewise, the addition of the muscarinic antagonist atropine and the corresponding antigenic peptide could abolish this effect (Fig. 3).

**DISCUSSION**

Myocardial damage triggered the genesis of autoantibody. In this study, we definitively demonstrated that myocardial damage itself, in two disparate models of heart failure and cardiomyopathy, was able to trigger the occurrence of an autoimmune reaction, resulting in the genesis of antireceptor autoantibodies in the absence of any additional interfering factors. In connection with the aforementioned clinical observations (1–10), although our results with rats may have some species-related difference, they could lend support to the view that the occurrence of abnormal antireceptor antibodies is not restricted to
DCM, but can also occur in relation to cardiac pathologic changes due to some other heart diseases. In support of our experimental findings, Peukert et al. (15) reported the occurrence of anticardiac receptor autoantibodies and their correlation with a clinical manifestation in patients with hypertrophic cardiomyopathy.

**Biologic property of generated autoantibodies.** In both experimental models of this study, it was shown that the

**Figure 1.** Changes in the frequency of the occurrence of both autoantibodies in the sera, before (pre) and after (post) four weeks of treatment. AB = aortic banding group (n = 24); ADR = adriamycin group (n = 24); Control = control of both experimental groups (n = 48). **p < 0.01 versus control group.

**Figure 2.** Time course of genesis of both autoantibodies in terms of changes in titers of autoantibodies after aortic banding (AB) (A) and adriamycin (ADR) (B) treatment (only sera positive for autoantibodies are shown).
autoantibodies induced by the cardiac remodeling process have pharmacologic and physiologic effects similar to those seen in human autoantibodies and rabbit antipeptide antibodies. These suggest that the autoantibodies generated due to cardiac structural and functional changes possess a biologic function similar to that in patients with DCM and, thus, can contribute to exacerbation of cardiac pathologic changes and dysfunction. Although beta1-adrenoceptor and M2 receptor antibodies have the opposite functional effects, it is difficult to judge the overall effect on cardiac function, because the effects of antibodies are dependent not only on their concentrations, but also on their affinities and avidities in antigen-antibody interactions. For example, the concentration of autoantibodies needed to affect the beta1-adrenoceptor is pharmacologically 10 times weaker than that needed to affect the M2 receptors (3). It is currently

Figure 3. Chronotropic effects on cultured neonatal rat cardiomyocytes induced by sera positive for the beta1-adrenoceptor (A) or the M2 receptor (B). These effects were respectively abolished by the addition of both receptor antagonists and pre-incubation with both antigenic peptides. PS = positive sera.
unknown whether these antireceptor autoantibodies are just a disease marker or whether they are disease causing, although there is increasing evidence supporting that antireceptor autoantibodies may be involved in the pathogenesis of idiopathic DCM (4,5).

Mechanisms responsible for the genesis of autoantibodies. So far, there are only a few reports suggesting that the production of anticardiac receptor autoantibodies might be related to the virus-induced cardiac damage and cross-reacting antigens (16,17). However, in our experimental models, neither the virus infection nor the cross-reacting antigens were involved. Nevertheless, the two models of heart failure in the rat in this study are both associated with myocyte necrosis and lysis, so we can assume that, in our experiment, the cardiac myocyte necrosis and lysis, along with structural and functional cardiac changes, may have affected immunologic function, as evidenced by the imbalance between the helper and cytotoxic T cells, and thus resulted in the genesis of autoantibodies. Although the trigger for autoantibody production may be myocyte necrosis (18), continued generation of autoantibodies may be caused by triggering of the neuroendocrine axis (19).

Characteristic time course of the generation of autoantibodies. In this study, both types of autoantibodies generated during the myocardial remodeling process showed a characteristic self-growth and time-course decline. Our observations demonstrated that the existence of both autoantibodies lasted for only a short period, with the titers gradually tapering in about two to three months. An immunosuppressive effect due to a neurohormonal disorder, occurring in the advanced stage of heart failure, may lead to the disappearance of the antireceptor antibodies (20). The regular growth and decline pattern of the autoantibodies could account for the clinical observation that positive sera for antireceptor autoantibodies are only detectable in a subgroup of patients with DCM and other heart diseases associated with heart failure. Although these autoantibodies only exist for a short period during the whole cardiac pathologic process, the antireceptor autoantibodies can indeed be pathophysiologically important in the development of heart diseases. Clinically speaking, therefore, the negative findings of antireceptor autoantibodies in the sera of patients with DCM or other heart diseases may not necessarily imply that there is no autoimmune reaction involved in the pathogenesis throughout the whole pathologic process. In concert with our findings, it was reported, clinically, that autoantibodies against the adenine nucleotide translocator exist in the sera of patients with DCM, only for a short period of about two to three months (20). Moreover, Caforio et al. (21) reported that cardiac autoantibodies in patients with DCM become undetectable with disease progression.

It is obvious that many gaps still remain in the current knowledge of the occurrence of autoantibodies directed against cardiac receptors, in relation to myocardial pathologic changes. Nevertheless, our preliminary observations may open new insights into the mechanism that involves the immunoreaction in the pathogenesis or pathologic role of some heart diseases, and may be the starting point for future new studies.

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REFERENCES