Myocardial Injury Due to G-protein Coupled Receptor-autoimmunity

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SUMMARY

One of the main mechanisms for dilated cardiomyopathy is likely to be autoimmune mediated myocardial damage. So far, a variety of autoantibodies have been detected against a number of putative autoantigens in the sera of patients with dilated cardiomyopathy. A growing body of studies have confirmed that autoantibodies against the second extracellular loop of β_1 -adrenoceptors and M2-muscarinic receptor are present in 30–40% of patients with dilated cardiomyopathy. These anti- β_1 -adrenoceptor and anti-M2-muscarinic receptor antibodies can not only decrease the binding sites of antagonist but also recognize the target receptors. Moreover, these two autoantibodies possess an 'agonist-like' stimulatory effect on the target receptors. In order to elucidate whether the autoantibodies against these autoimmune epitopes play an important role in the pathogenesis of dilated cardiomyopathy, we immunized rabbits over a period of one year with synthetic peptides corresponding to the second extracellular loop of the β_1 -adrenoceptor and the M2-muscarinic receptor. These peptides induced morphological changes in the heart similar to those found in dilated cardiomyopathy. These clinical and experimental findings suggest that these receptor autoantigens are of pathogenic importance in the development of dilated cardiomyopathy *in vivo*. (Jpn Heart J 1998; 39: 261–274)

Key words: Autoantibody, G-protein-coupled receptor, Dilated cardiomyopathy, Myocarditis, Experimental cardiomyopathy, Synthetic peptide

THE heart has a susceptibility to immune-mediated injury. It is known that myocytes may be injured during or after an immune response to endogenous or exogenous cardiac antigens. Immune-mediated myocyte injury may be reversible and manifest as a transient depression of ventricular function and/or electrical instability, or it may be irreversible and lead ultimately to myocyte necrosis, or apoptosis.¹⁾

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Mechanisms of immune-mediated myocyte injury may be classified into humoral and cellular types. Humoral immunity requires recognition of an epitope by antibody molecules expressed by B cells and depends on circulating antibodies to initiate injury. Several cardiac diseases including postmyocardial infarction syndrome, dilated cardiomyopathy, viral myocarditis and doxorubicin cardiotoxicity are occasionally accompanied by the deposition of antibodies within the myocardium, particularly on the sarcolemma.²⁾ Furthermore, many studies have demonstrated the presence of heart-reactive antibodies in 13% to 95% of patients with various myocardial diseases such as dilated cardiomyopathy, myocarditis, hypertrophic cardiomyopathy or Chagas' disease (Table I).³⁻¹⁶⁾ One of the mechanisms of myocyte injury mediated by humoral immunity is the activation of complement via the classic pathway involving binding of the C1 component by an antigen-antibody complex on the surface of the target cell. A cascade of reactions is initiated that results in the formation of the membrane attack complex which is arranged into a plasma membrane pore.¹⁷⁾ Another mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC). Many cell types that bear receptors for the Fc portion of IgG cause the lysis of target cells coated with IgG antibody. This interaction involves the Fc receptors of the killer cells, which engage the Fc portion of the antibody coating target cell. ADCC can be mediated by a variety of cell types bearing Fc-IgG receptors. These include neutrophils, eosinophils, macrophages, and natural killer cells. In some cases,

Table I. Autoantibodies against Cardiac Tissue in Myocardial Diseases

Antibody to	Prevalence (%)					Method	Reference
	DCM	MC	HCM	Chagas	NC		
Mitochondria (M7)	31	13	33	ND	0	ELISA	Klein <i>et al.</i> ³⁾ 1984
Adenine nucleotide translocator	95	ND	ND	ND	0	SPRIA	Schultheiss <i>et al.</i> ⁴⁾ 1985
Laminin	78	73	ND	ND	6	ELISA	Wolff <i>et al.</i> ⁵⁾ 1989
Myosin	ND	42	ND	ND	2.5	ELISA	Lauer <i>et al.</i> ⁶⁾ 1994
Heart	20	59	ND	ND	0	immunofluorescence	Neumann <i>et al.</i> ⁷⁾ 1990
Heart	26	ND	ND	ND	3.5	immunofluorescence	Caforio <i>et al.</i> ⁸⁾ 1990
Heart shock protein 60	85	ND	ND	ND	ND	SDS-PAGE & Western	Latif <i>et al.</i> ⁹⁾ 1993
Heart shock protein 60	10	ND	ND	ND	1	2D immunoblot	Portig <i>et al.</i> ¹⁰⁾ 1997
Heart shock protein 70	14	ND	ND	ND	3		
β -receptor	46-75	ND	ND	ND	0-18	ligand binding inhibition	Limas <i>et al.</i> ¹¹⁾ 1989
β_1 -receptor	31	ND	ND	ND	12	ELISA	Magnusson <i>et al.</i> ¹²⁾ 1990
β_1 -receptor	ND	ND	ND	36	12	ELISA	Rosenbaum <i>et al.</i> ¹³⁾ 1994
Muscarinic-2 receptor	38	ND	ND	ND	8	ELISA	Fu <i>et al.</i> ¹⁴⁾ 1993
Muscarinic-2 receptor	ND	ND	ND	53	0	ELISA	Goin <i>et al.</i> ¹⁵⁾ 1997

DCM = dilated cardiomyopathy; ELISA = enzyme-linked immunoabsorbent assay; HCM = hypertrophic cardiomyopathy; MC = myocarditis; NC = normal control; ND = not done; SPRIA = solid-phase radioimmunoassay; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis.

Table II. Cellular Immunity in Dilated Cardiomyopathy (DCM) and in Myocarditis (MC)

Reference	Abnormality in cellular immunity
Flowles <i>et al.</i> ¹⁹⁾ 1979	Reduced suppressor T-cell activity in DCM
Eckstein <i>et al.</i> ²⁰⁾ 1982	Reduced suppressor T-cell activity in DCM and MC
Anderson <i>et al.</i> ²¹⁾ 1982	Deficient natural killer cell activity in DCM
Sanderson <i>et al.</i> ²²⁾ 1985	High percentage of helper T-cells and high helper/suppressor ratio in DCM
Rönnblom <i>et al.</i> ²³⁾ 1991	Increased HLA-DR positive T-cells in DCM
Caforio <i>et al.</i> ²⁴⁾ 1990	Inappropriate expression of MHC class II molecule
Herskowitz <i>et al.</i> ²⁵⁾ 1990	Increased expression of MHC class I and II on myocytes in MC
Koike <i>et al.</i> ²⁶⁾ 1989	Enhanced IL-2 production and reduced expression of IL-2 receptor of lymphocytes in DCM
Limas <i>et al.</i> ²⁷⁾ 1995	Elevated soluble IL-2 receptor levels in DCM
Seko <i>et al.</i> ²⁸⁾ 1992	Expression of ICAM-1 on the ventricular tissue in acute MC
Toyozaki <i>et al.</i> ²⁹⁾ 1993	Expression of ICAM-1 on cardiac myocytes in chronic MC
Seko <i>et al.</i> ³⁰⁾ 1995	Infiltration of perforin-expressing killer cells, enhanced expression of HLA class I and ICAM-1 in the myocardium of DCM. Restricted usage of T-cell receptor V α -V β genes in infiltrating cells in the heart of patients with MC and DCM.
Badorff <i>et al.</i> ³¹⁾ 1997	Expression of perforin and T-cell intercellular antigen-1 in hearts with DCM.

ICAM = intercellular adhesion molecule; IL = interleukin; MHC = major histocompatibility complex.

antibodies directed against cell surface receptors impair or dysregulate function without causing cell injury or inflammation. However, there is no convincing evidence available suggesting that humoral immunity plays a dominant role in the mechanism of myocyte injury.¹⁸⁾

Cellular immunity also plays a role in the mechanism underlying cardiac dysfunction. Cellular immunity is initiated by recognition of an immunogenic epitope by antigen-specific T cells. This initial recognition and activation is effected by helper T cells and requires the presence of histocompatible antigen-presenting cells. Presentation of myocardial antigen to T cells by antigen-presenting cells can result in the clonal expansion and differentiation of cytotoxic T cells, stimulated by the release of immune cytokines. The consequent myocyte necrosis can result in fibrosis.¹⁸⁾ In dilated cardiomyopathy and myocarditis, abnormalities in cell-mediated immunity have been clinically demonstrated by the findings of altered lymphocyte function, altered relative proportions of lymphocyte subsets, activated immune cytokine systems, the inappropriate expression of the major histocompatibility complex on cardiac tissue, and expression of adhesion molecules on cardiac myocytes (Table II).¹⁹⁻³²⁾

The relative contribution of cellular and humoral immune disturbances to the pathogenesis of myocyte injury in various heart diseases has not been determined.

AUTOANTIBODIES AGAINST G-PROTEIN COUPLED CARDIOVASCULAR RECEPTORS

Disease specificity of autoantibodies: There is an increasing amount of information available supporting the finding that one of the targets of autoimmune attack in dilated cardiomyopathy could be functionally important G-protein-coupled cardiac receptors such as the β -adrenoceptor and M2-muscarinic receptor. Limas *et al.* demonstrated that anti- β -adrenoceptor antibodies were present in about 30–40% of patients with dilated cardiomyopathy using a ligand-binding inhibition assay, compared to about 15% of those with other cardiac disease and none in the normal controls.¹¹⁾ Magnusson *et al.* reported that high reactivity (31%) was seen toward a synthetic β_1 -peptide corresponding to the second extracellular loop of β_1 -adrenoceptor with little reactivity (0.5%) toward a synthetic β_2 -peptide corresponding to the second extracellular loop of β_2 -adrenoceptor in patients with dilated cardiomyopathy, and none of the ischemic heart disease patients recognized the synthetic β_1 -peptide.¹²⁾ Fu *et al.* studied these autoantibodies against the second extracellular loop of β_1 -adrenoceptor in patients with dilated cardiomyopathy from different geographic origins such as Japan and Sweden and found a similar spectrum of circulating anti- β_1 -adrenoceptor autoantibodies.¹⁶⁾ Moreover, Fu *et al.* in another study demonstrated the presence of functional autoantibodies against the second extracellular loop of the M2-muscarinic receptor in 14 of 36 patients (38%) with dilated cardiomyopathy.¹⁴⁾ Chagas' disease could arise as a result of parasite-induced autoimmune response. Recent studies have shown a high prevalence of autoantibodies against the second extracellular loop of β_1 -adrenoceptor (36%) and M2-muscarinic receptor (53%) in Chagas' disease.^{13,15)} The prevalence of these anti-receptor autoantibodies is similar to that in dilated cardiomyopathy.

The disease-specificity of the presence of circulating anti-receptor autoantibodies in dilated cardiomyopathy was studied by (1) comparing different patient groups using the same antigenic receptor peptide and (2) comparing the immune responses to different antigenic receptor peptides in the same patient group, such as those with dilated cardiomyopathy. Fu *et al.* reported anti- β_1 -adrenoceptor

Table III. Five Peptides Used for Immunoassay

Receptor	Position	Sequence
β_1	197–222	H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C
β_2	172–197	H-W-Y-R-A-T-H-G-E-A-I-N-C-Y-A-N-E-T-C-C-D-F-F-T-N-Q-C
α_1	192–218	G-W-K-E-P-V-P-P-D-E-R-F-C-G-I-T-E-E-A-G-Y-A-V-F-S-S-V
M2	169–193	V-R-T-V-E-D-G-E-C-Y-I-Q-F-F-S-N-A-A-V-T-F-G-T-A-I-C
AT1(AT1 α)	164–190	I-H-R-N-V-F-F-I-E-N-I-T-V-C-A-F-H-Y-E-S-Q-N-S-T-L

β_1 and $\beta_2 = \beta_1$ and β_2 -adrenoceptors; $\alpha_1 = \alpha_1$ -adrenoceptor; AT1(AT1 α) = angiotensin II-1 receptor; M2 = muscarinic receptor-2.

autoantibodies and anti-M2-muscarinic receptor autoantibodies in patients with dilated cardiomyopathy but not in patients with malignant hypertension.³³ In contrast, in as high as 64% of patients with malignant hypertension, circulating autoantibodies against the second extracellular loop of the α_1 -adrenergic receptor were observed. Further studies were performed to see whether target receptors for anti-receptor autoantibodies in dilated cardiomyopathy are restricted to two functionally important receptors such as the β_1 -adrenoceptor and M2-muscarinic receptor or can be other cardiovascular receptors which share a similar structure. By virtue of synthetic peptides, we investigated a series of G-protein coupled cardiovascular receptors which are of importance for cardiovascular function. Synthetic peptides corresponding to the sequences of the second extracellular loops of human β_1 -adrenoceptor, β_2 -adrenoceptor, α_1 -adrenoceptor, M2-muscarinic receptor and angiotensin II receptor subtype I (AT1 receptor) were used as antigens in an enzyme immunoassay (Table III). We examined the frequencies of anti-receptor autoantibodies against these five receptors in sera of 28 patients with dilated cardiomyopathy, 18 patients with myocarditis, 23 patients with hypertrophic cardiomyopathy and 37 patients with essential hypertension. Sera from 23 age-matched healthy subjects served as the normal control. Nine sera from patients with dilated cardiomyopathy (32%), 6 from patients with myocarditis (33%) and 2 from healthy subjects (9%) recognized the synthetic β_1 -peptide. Ten sera from patients with dilated cardiomyopathy (36%), 2 from patients with myocarditis (11%) and 3 from healthy subjects (13%) recognized the synthetic M2 peptide. There were significant differences between dilated cardiomyopathy patients and healthy subjects in terms of the frequency of anti- β_1 -adrenoceptor and anti-M2-muscarinic receptor antibodies. There was also a significant difference between myocarditis patients and healthy subjects in terms

Table IV. Autoantibodies against Various Cardiac Receptors
Coupled to G-protein as Detected by ELISA

	β_1	β_2	α_1	M2	AT1
DCM (<i>n</i> = 28) positive	9 (32%)**	2 (7%)	4 (14%)	10 (36%)*	1 (4%)
Myocarditis (<i>n</i> = 18) positive	6 (33%)**	2 (11%)	3 (17%)	2 (11%)	3 (17%)
HCM (<i>n</i> = 23) positive	3 (13%)	1 (4%)	2 (8%)	5 (22%)	0 (0%)
HT (<i>n</i> = 37) positive	3 (8%)	7 (19%)	5 (14%)	1 (3%)	0 (0%)
NC (<i>n</i> = 23) positive	2 (9%)	2 (9%)	2 (9%)	3 (13%)	3 (13%)

***p* < 0.5, **p* < 0.1 vs NC, AT1 = angiotensin II-1; DCM = dilated cardiomyopathy; ELISA = enzyme-linked immunoabsorbent assay; HCM = hypertrophic cardiomyopathy; HT = hypertension; M2 = muscarinic receptor-2; NC = normal controls.

of the frequency of anti- β_1 -adrenoceptor antibodies. However, there were no significant differences among hypertrophic cardiomyopathy, essential hypertension, and normal controls in terms of the frequencies of anti- β_1 -, anti- β_2 -, anti- α_1 -adrenoceptors, anti-M2-muscarinic receptors and anti-AT1 receptors antibodies (Table IV). Our results strongly suggest that patients with dilated cardiomyopathy may have a specific spectrum of anti-receptor autoantibodies, and that the high frequency of anti-receptor autoantibodies in dilated cardiomyopathy is restricted to the most predominant cardiac receptors, namely β_1 -adrenoceptors and M2-muscarinic receptors.³⁴⁾ The frequency of anti- β_1 -adrenoceptor antibody in acute myocarditis was similar to that in dilated cardiomyopathy, suggesting a possible link between dilated cardiomyopathy and the preceeding viral myocarditis. Some patients with viral myocarditis have later on had chronic myocardial dysfunction; conversely, patients with dilated cardiomyopathy have often had a related history of an antecedent flu-like illness. The evolution of myocardial damage in viral myocarditis occurs in two phases. The acute phase is characterized by viral invasion into the myocardium. After this stage, the myocardium is infiltrated by inflammatory cells and there is activation of the immune system, including the production of heart-reactive antibodies. This phase is associated with varying degrees of myocardial damage.

Functional consequences of autoantibodies: It has been demonstrated that affinity-purified autoantibody against the second extracellular loop of β_1 -adrenoceptor from patients with dilated cardiomyopathy had the ability to recognize the β_1 -adrenoceptor in human myocardium, and to inhibit the binding of radiolabeled β blockade to the β_1 -adrenoceptor without a change in the dissociation constant.^{11,12)} These autoantibodies were also shown to increase the beating frequency of isolated myocytes. This positive chronotropic effect of the antibodies was completely neutralized by the addition of increasing concentrations of a β_1 -selective blockade.³⁵⁾ Addition of isoproterenol to autoantibody-stimulated myocytes resulted in only a small increase in beating frequency and does not cause desensitization. This phenomenon was in accordance with the observation that autoantibodies block cAMP accumulation induced by isoproterenol.^{11,35)} On the mapping of epitope with short synthetic overlap peptides of the second extracellular loop of the β_1 -adrenoceptor, the positive chronotropic response of cultured rat cardiomyocytes to the anti- β_1 -adrenoceptor autoantibodies was inhibited by a particular neutralized overlapping peptide, namely A-R-R-C-Y-N-D (residues 206–212).³⁶⁾

On the other hand, we have demonstrated a specific localization of M2-muscarinic receptors in human myocardium from dilated cardiomyopathy using affinity-purified autoantibodies against the second extracellular loop of M2-muscarinic receptor from patients with dilated cardiomyopathy. This finding suggests

that anti-M2-muscarinic receptor antibodies can interact with their target receptors in heart tissue. Moreover, these affinity-purified anti-M2-muscarinic receptor antibodies induce a decrease in the maximal binding sites (B_{max}) of radioligand M2-muscarinic receptor antagonists, with an increase in dissociation constant.¹⁴⁾ Preincubation with atropine abolishes the inhibitory effect of anti-M2-muscarinic receptor antibodies on receptor binding, whereas carbachol appears to have no effect on the activity of autoantibodies.¹⁴⁾ These anti-M2-muscarinic receptor antibodies, affinity-purified from sera of patients with dilated cardiomyopathy, are able to mimic muscarinic agonist stimulation as demonstrated by a negative chronotropic effect on cultured cardiomyocytes. This negative chronotropic effect is abolished by the addition of muscarinic antagonist atropine.³⁷⁾ Affinity-purified antibody against the shorter synthetic peptide of the second extracellular loop of M2-muscarinic receptor (amino acids 172–181. V-E-D-G-E-C-Y-I-Q-F) was able to recognize M2-muscarinic receptors and had a negative chronotropic effect on rat cardiomyocytes. However, this antibody was unable to inhibit radioligand binding on the heart muscarinic receptors. This fact indicates that anti-M2-muscarinic receptor antibodies in patients with dilated cardiomyopathy are heterogenous and recognize two epitopes in the heart.³⁷⁾

Because anti- β_1 -adrenoceptor antibodies and anti-M2-muscarinic receptor antibodies have a reciprocal effect on myocyte function, it is difficult to judge the overall effects of these anti- β_1 -adrenoceptor antibodies and anti-M2-muscarinic receptor antibodies on cardiac function or on the evolution of myocardial damage. Previous results have demonstrated that the concentration of autoantibodies needed to pharmacologically affect the β_1 -adrenoceptors is ten times lower than that needed to affect the M2-muscarinic receptor.¹⁴⁾

Correlation between clinical states and antibody titers: It is expected that patients with more severe cardiac dysfunction have higher antibody titers if the antibody plays a causative role in the disease process. Limas and colleagues observed a rough correlation between the degree of left ventricular systolic dysfunction and autoantibody titers.³⁸⁾ However, we could not find a significant correlation between the titers of antibodies against the second extracellular loop of β_1 -adrenoceptor and M2-muscarinic receptor and the clinical severity of the patient with DCM.³⁴⁾ Accordingly, a wider spectrum of dysfunction is needed before meaningful conclusions can be drawn.

Caforio *et al.* reported that the frequency of cardiac-specific autoantibodies was higher among relatives of patients with dilated cardiomyopathy than in controls (20% vs 3.5%).³⁹⁾ Limas *et al.* reported a high prevalence of anti- β -adrenoceptor antibodies (68%) in affected compared to unaffected (29%) members in an Ohio family with dilated cardiomyopathy.³⁸⁾ These reports suggest that the presence of dilated cardiomyopathy can be detected before clinical disease

becomes manifest.

Anti- β_1 -adrenoceptor antibodies are extractable by immunoabsorption. Dörffel *et al.* reported that immunoabsorption was effective in reducing the titers of anti- β_1 -adrenoceptor antibodies and resulted in a marked improvement in hemodynamics in patients with severe dilated cardiomyopathy.⁴⁰⁾

Further study will be necessary to determine whether longitudinal changes in clinical states correlate with the antibody titers.

EXPERIMENTAL CARDIOMYOPATHY INDUCED BY AUTOIMMUNITY AGAINST G- PROTEIN-COUPLED CARDIOVASCULAR RECEPTORS

It has been shown that the peptides corresponding to the second extracellular loop of the β_1 -adrenoceptor and the M2-muscarinic receptor contain both T- and B-cell epitopes so that they can be used as immunogens without any previous coupling to a carrier peptide.^{37,41,42,43)} Since the antibodies recognize functional B-cell epitopes on the target receptors and, in view of the complete conservation of the sequence loops of M2-muscarinic receptors in three mammalian species (pig, rat, man) and of that of β_1 -adrenoceptors in man and rodents, it can be assumed that the same sequence is present in the respective receptors of rabbits. These receptor peptides can thus be regarded as excellent autoantigens in rabbits. Magnusson *et al.* showed that antibodies with high titers can be obtained from the sera of rabbits immunized by a synthetic peptide corresponding to the second extracellular loop of the β_1 -adrenoceptor and that these antibodies can be bound to the β_1 -adrenoceptor of rabbit myocardium.¹²⁾ Similarly, Fu *et al.* recognized that antibodies with high titers can be obtained from sera of rabbits immunized by a synthetic peptide corresponding to the second extracellular loop of the M2-muscarinic receptor and that these anti-M2 muscarinic receptor antibodies can be bound to the M2 muscarinic receptors on the sarcolemma and t-tubules of rat cardiomyocytes.^{14,37,44)} Moreover, these rabbit anti- β_1 -peptide antibodies have a positive chronotropic effect and rabbit anti-M2 peptide antibodies have a negative chronotropic effect just like affinity-purified antibodies from sera of patients with dilated cardiomyopathy.^{12,14,33,35,37,41)}

In order to elucidate whether the antibodies against these autoimmune epitopes play an important role in the pathogenesis of cardiomyopathy, we immunized rabbits over a period of 1 year with synthetic peptides corresponding to the second extracellular loop of the β_1 -adrenoceptor and the M2-muscarinic receptor (Table III).

In the sera from both β_1 and M2 immunized groups, high titers of anti- β_1 or anti-M2 peptide antibodies were found throughout the study period. The myocardial receptor density of both immunized groups displayed a strong trend to-

Table V. Body Weight and Heart Weight in Both Immunized Rabbits and Control Rabbits (mean $\pm$ S.D)

	Body weight (initial) (kg)	Body weight (final) (kg)	Heart weight (g)
β_1 group ($n = 6$)	2.40 ± 0.22	4.09 ± 0.49	$10.1 \pm 1.3^*$
M2 group ($n = 6$)	2.50 ± 0.11	4.19 ± 0.52	9.6 ± 1.7
Control group ($n = 6$)	2.39 ± 0.193	3.61 ± 0.345	8.3 ± 1.31

* $p < 0.05$ vs corresponding control.

Figure 1. Transverse sections of the heart (hematoxylin and eosin stain). **a:** Heart from a control rabbit showing normal chamber size and wall thickness. **b:** Heart from the β_1 immunized rabbit showing mild dilatation of the right ventricle. **c:** Heart from the β_1 immunized rabbit which died after 9 months of immunization showing marked dilatation of the right ventricle with conspicuous wall thinning. **d:** Heart from the M2 immunized rabbit showing dilatation of the right ventricle with wall thinning (scale: 1 mm).

ward receptor up-regulation without a change in the dissociation constant. The heart weights of the β_1 immunized group were significantly increased, compared to those of the control rabbits (Table V). Both immunized groups displayed significantly enlarged ventricles and thinner walls, compared to those of the control rabbits. In contrast to the β_1 immunized group, which showed enlarged cavities in both left and right ventricles, the M2 immunized group was mainly

Table VI. Anatomic Measurements of Hearts from Both Immunized Rabbits and Control Rabbits at the End of Experiments (mean $\pm$ S.D.)

	Control group (n = 6)	β_1 group (n = 8)	M2 group (n = 6)
Wall thickness (mm)			
Right ventricles	1.10 $\pm$ 0.118	0.62 $\pm$ 0.187***	0.68 $\pm$ 0.079***
Interventricular septum	2.75 $\pm$ 0.567	2.44 $\pm$ 0.637	2.96 $\pm$ 0.262
Left ventricles	3.16 $\pm$ 0.488	2.53 $\pm$ 0.640	2.95 $\pm$ 0.414
Cavity dimension (mm)			
Right ventricles	2.85 $\pm$ 1.389	4.87 $\pm$ 1.797**	5.7 $\pm$ 1.216***
Left ventricles	9.04 $\pm$ 0.951	11.60 $\pm$ 1.946**	8.89 $\pm$ 1.397 ^a
Cavity area (mm ²)			
Right ventricles	42.2 $\pm$ 12.61	63.8 $\pm$ 32.42	67.3 $\pm$ 24.07**
Left ventricles	68.5 $\pm$ 18.66	69.8 $\pm$ 16.36	68.6 $\pm$ 17.43
Total ventricular wall area (mm ²)	158.6 $\pm$ 12.85	158.4 $\pm$ 23.55	160.5 $\pm$ 15.14

** p < 0.05; *** p < 0.01 vs Control; ^a p < 0.05 vs β_1 group.

Figure 2. Light micrographs of the heart (original magnification $\times$ 150, hematoxylin and eosin stain). **a:** Heart from a control rabbit showing intact and normal myocardial cells and no inflammatory response. **b:** Heart from the β_1 immunized rabbit showing focal degeneration and necrosis of myocardial cells with infiltration of mononuclear cells. **c:** Heart from the β_1 immunized rabbit which died after 9 months of immunization showing extensive interstitial fibrosis with infiltration of mononuclear cells. **d:** Heart from the M2 immunized rabbit showing focal degeneration and necrosis of myocardial cells with infiltration of mononuclear cells.

affected in the right ventricles (Figure 1, Table VI). In light microscopic examinations, the hearts of both immunized groups displayed significant alterations as shown by the multifocal degeneration and necrosis of myocardial cells, together with the infiltration of inflammatory cells. These inflammatory cells are mainly lymphocytes and macrophages. These changes were present in the walls of both ventricles and in the interventricular septum, but the right ventricle was more generally affected than the left ventricle (Figure 2). Electron microscopic examinations of the heart of both immunized groups demonstrated focal myofibrillar lysis, loss of myofilament, mitochondrial swelling and condensation, sarcoplasmic vacuolation, and deposition of dense granules in the sarcoplasm and myofibrils. Rabbit IgG was localized on the sarcolemma of cardiomyocytes in both immunized groups.⁴⁵⁾

Our results suggest that synthetic peptides corresponding to the second extracellular loops of the human β_1 -adrenoceptor and M2-muscarinic receptor are able to induce morphological changes in rabbits following one year of active immunization. The changes, resembling those of dilated cardiomyopathy in humans, thereby suggest that these receptor autoantigens are of pathogenic importance in the development of cardiomyopathy *in vivo*.

In histochemical studies, CD4⁺ and CD8⁺ T-cells and macrophages were recognized in the myocardium of β_1 and M2 immunized rabbits. Moreover, both major histocompatibility complex class I and class II were expressed on the myocardium (unpublished data). Thus, the following mechanisms of myocardial injury in rabbits immunized by β_1 and M2 peptides can be proposed; (1) antibody-dependent cell-mediated cytotoxicity (ADCC), (2) T cell-mediated cytotoxicity, and (3) antibody-mediated cellular dysfunction. In spite of high levels of anti- β_1 -adrenoceptor or anti-M2-muscarinic receptor autoantibodies in the sera of rabbits immunized with one of the peptides, myocardial injury in β_1 immunized rabbits was severe compared to that in M2 immunized rabbits. These two anti-peptide antibodies exhibit 'agonist-like' stimulation of the target receptors. Moreover, the chronotropic effect of anti- β_1 -adrenoceptor autoantibody is about ten times greater than that of anti-M2 muscarinic receptor autoantibody. Based on these findings, it is assumed that myocardial injury in β_1 immunized rabbits may be mainly induced by mild or moderate stimulation of β_1 -adrenoceptor for one year, and that in M2 immunized rabbits it may be mainly induced by ADCC and/or T cell-mediated cytotoxicity.

FURTHER PERSPECTIVES

Although many studies have been performed to explain the relationship between receptor-autoimmunity and dilated cardiomyopathy, there still exists a

big gap between hypothesis and reality. Further studies should focus on the properties of autoantibodies both *in vitro* and *in vivo*, the molecular and cellular mechanisms behind the action of autoantibodies, the modulating mechanisms among different autoantibody actions, and the reproduction of dilated cardiomyopathy using pathogenic putative autoantigens. We hope that a breakthrough in the understanding of the relationship between receptor-autoimmunity and dilated cardiomyopathy will take place in the near future as it will certainly add a new therapeutic potential to the management of dilated cardiomyopathy.

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