Autoimmunity in Idiopathic Dilated Cardiomyopathy
Characterization of Antibodies Against the $\beta_1$-Adrenoceptor With Positive Chronotropic Effect

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Background Autoantibodies against the $\beta_1$-adrenoceptor have been detected in the sera of patients with idiopathic dilated cardiomyopathy (DCM). The mechanisms by which these autoantibodies can alter normal receptor function are investigated, and the results are interpreted in the light of the beneficial effects of $\beta_1$-blockade in some of these patients.

Methods and Results Autoantibodies against the $\beta_1$-adrenoceptor, affinity purified from sera of patients with idiopathic DCM, were analyzed in a functional test system of spontaneously beating neonatal rat heart myocytes. Antibodies from rabbits immunized with peptides derived from the amino acid sequence of this receptor were also analyzed. Autoantibodies against the second extracellular loop increased the beating frequency of isolated myocytes in a concentration-dependent manner, to approximately 80% of maximal isoproterenol stimulation. Rabbit anti-peptide antibodies against the second extracellular loop increased the beating frequency correspondingly. Autoantibodies and rabbit anti-peptide antibodies against the second extracellular loop were able to immunoprecipitate the unliganded receptor but not the antagonist-occupied receptor. In contrast, rabbit antibodies against the extracellular N-terminal sequence 34-57 of the $\beta_1$-adrenoceptor were able to immunoprecipitate both the unliganded and the antagonist-occupied receptor although with no effect on the beating frequency of myocytes. The positive chronotropic effect of the antibodies was completely neutralized both by the addition of increasing concentrations of the $\beta_1$-selective antagonist bisoprolol and by preincubation with the peptide corresponding to the second extracellular loop. The antibody-induced increase in beating frequency remained unchanged for more than 6 hours. This should be compared with the isoproterenol-stimulated beating frequency, which undergoes desensitization within 60 minutes. Addition of isoproterenol to autoantibody-stimulated myocytes resulted in only a small increase in beating frequency and did not cause desensitization. Antibodies had only a marginal effect on cyclic AMP production of stimulated cardiomyocytes compared with the 10-fold increase obtained after stimulation with isoproterenol.

Conclusions The second extracellular loop of the $\beta_1$-adrenoceptor is a specific target for antibodies with stimulatory activity detected in patients with idiopathic DCM. The antibodies have a positive chronotropic effect on isolated rat heart myocytes. Autoantibody stimulation does not cause the normal agonist-induced desensitization phenomena of the effector system. These findings could contribute to our understanding of the pathophysiological mechanisms of the autoantibodies and of the beneficial effect of $\beta_1$-blocking agents in the treatment of patients with idiopathic DCM. (Circulation. 1994;89:2760-2767.)

Key Words • antibodies • cardiomyopathy • myocytes • peptides

Diastolic dilated cardiomyopathy (DCM) is defined as a heart muscle disease without a known cause.1 During the past few years, considerable interest has been focused on the infections-immune hypothesis. It has been claimed that various infectious agents, such as viruses, trigger an autoimmune reaction, which progressively destroys myocardial tissue and leads to DCM.2 Several findings, such as the imbalance between helper and cytotoxic T cells,3 the inappropriate expression of the major histocompatibility complex on cardiac tissues,4 and the existence of circulating organ-specific autoantibodies, support this hypothesis.5-7 More recently, it has been shown that autoantibodies against the cardiac $\beta$-adrenergic receptors are present in the sera of patients with DCM.8-10 In our earlier studies, it was possible to localize an epitope recognized by anti-$\beta$-adrenergic receptor autoantibodies on an amino acid sequence corresponding to the postulated second extracellular loop of the human $\beta_1$-adrenergic receptor.9 It has been reported that a serum $\gamma$-globulin fraction from patients with myocarditis and DCM had a positive chronotropic effect on cultured neonatal rat heart myocytes and that this effect could be blocked by $\beta_1$-selective antagonists but not by a $\beta_2$-selective antagonist.11,12

The purpose of the present study was to see whether the epitopic target of the autoantibodies, which have a positive chronotropic effect, could be identified as the second extracellular loop. Autoantibodies from patients with idiopathic DCM were affinity purified on a peptide corresponding to the sequence of this loop. Concomitantly, rabbit antibodies were induced against the same...
peptide and tested for their potential to mimic the human autoantibodies by comparing their immunochemical properties, their pharmacological specificities, and their functional activities on cardiomyocytes in vitro.

Methods

Patients
All patients (six men and one woman; age, 55±9 years) had been clinically diagnosed as having idiopathic DCM. They all had been hospitalized for congestive heart failure and were treated with diuretics, digitalis, and angiotensin-converting enzyme inhibitors. None of the patients were receiving treatment with \( \beta \)-blockers, calcium blockers, or inotropic or anti-depressant drugs. Coronary artery disease was excluded based on coronary angiography, and no patient had a history of hypertension, valvular heart disease, diabetes, or any other endocirnal disorder or suspicion of alcohol or drug abuse. Acute or chronic myocarditis was excluded by the findings of right ventricular endomyocardial biopsy. All patients had signs of depressed left ventricular function (ejection fraction, 25±9%) as judged from noninvasive investigation by M-mode and two-dimensional echocardiography. The New York Heart Association functional class of the patients was 2.5±0.5.

Peptides

Two peptides, corresponding to the sequence of the extracellular N-terminal (V-P-A-S-P-P-A-S-L-L-P-A-S-S-P-E-P-L-S-Q-Q-W-C) (34-57) and of the second extracellular loop (H-W-W-R-A-E-S-D-E-A-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R) (197-222) of the human \( \beta \)-adrenergic receptor,15 were synthesized using the solid-phase method of Merrifield16 with an automated Applied Biosystems 430A peptide synthesizer. The peptides were desalted on a Biorad P6 desalting-grade molecular sieve using 0.1 mol/L Na2CO3 as eluent and stored in the same solvent at -20°C until use. The composition of the purified peptides was tested in an automatic Beckman amino acid analyzer.

Chemicals

(−)-Isoproterenol was purchased from Sigma Chemical Co, and bisoprolol and ICI 118.551 were the kind gifts of Merck AG and ICI Ltd, respectively. All other reagents were of analytical grade.

Immunization

Rabbits were immunized intracutaneously with 1 mg of free peptide emulsified in Freund's complete adjuvant. Four weeks after the primary injection, a booster injection of 0.1 mg peptide was given subcutaneously in Freund's incomplete adjuvant. Booster injections were given on weeks 9, 14, and 19. Bleedings were begun on week 5 and continued every second week. Rabbits were bled to death 20 weeks after the first injection.

Affinity Purification of Antibodies

Based on a sera-positive response in an enzyme immunoassay to peptide 197-222 of the \( \beta \)-adrenoceptor, sera from four patients with DCM were selected, pooled, and affinity purified (pool n=4). Positive sera from three additional patients with DCM were individually affinity purified (patients 5 through 7). The sera of immunized rabbits, described in the previous section, were also affinity purified. All sera were affinity purified in the following manner: the affinity column was made up by coupling the peptide 197-222 or the peptide 34-57 to CNBr-activated Sepharose, using the standard procedure. After purification of the \( \gamma \)-globulin fraction by 35% \((\text{NH}_{4})_{2}\text{SO}_{4}\) saturation, rabbit anti-peptide antibodies and autoantibodies from patients were adsorbed on the affinity column in physate-buffered saline (PBS), eluted with a 0.2-mol/L glycine buffer (pH 2.6), immediately neutralized in 1 mol/L Tris (pH 8.0), and dialyzed extensively against PBS (10 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4). Antibody concentrations were measured by optical density at 280 nm. The purified antibodies were stored at -20°C until use.

Enzyme Immunoassay

NUNC microtiter plates were coated with solutions of 50 \( \mu \)g/mL peptide in 0.1 mol/L Na2CO3, plus mercaptoethanol 1% for 1 hour at room temperature. After saturation of the wells with PBS supplemented with 3% skim-milk powder and 0.1% Tween 20, antibody dilutions in the same buffer were allowed to adsorb on the peptides for 1 hour at room temperature. The antibodies from patients with DCM and from immunized rabbits were revealed by successive 1-hour incubations at room temperature with rabbit biotinylated anti-human IgG antibodies and donkey biotinylated anti-rabbit IgG antibodies (diluted 1:1000 in saturation buffer) (Jackson Laboratories), respectively, followed by a streptavidin-peroxydase conjugate incubation at a 1:800 dilution (Jackson Laboratories) in the same buffer. After washing the wells with PBS, H2O2-ABTS substrate was added, and the optical density was measured at 405 nm with a Molecular Devices Vmax ELISA plate reader.

Immunoprecipitation

Membranes from Escherichia coli expressing the functional human \( \beta \)-adrenoceptor17 were prepared as previously described.9 A solubilized radioligand-receptor complex was prepared as described.16 After incubating the membranes with 200 pmol/L of the radioligand [125I]iodocyanopindolol ([125I]ICYP), the membranes were solubilized on ice for 1 hour with 2% digitonin in a 10 mmol/L Tris buffer (pH 7.2) containing 100 mmol/L NaCl. The free radioligand was separated from the complex by molecular sieving on a Sephadex G25 column equilibrated in a 0.1% digitonin solution of 10 mmol/L Tris (pH 7.2) and 150 mmol/L NaCl. The solubilized complex was then stored at -80°C until use. Twenty fentomoles of this complex was incubated with an equal volume of affinity-purified antibodies for 2 hours at room temperature before adding protein A-Sepharose 4B beads (Pharmacia), followed by further incubation for 1 hour at room temperature. The beads were washed extensively with a 20-mmol/L Tris buffer (pH 7.5) containing 1% Triton X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mol/L NaCl, 50 mmol/L KCl, and 1 mmol/L EDTA. Radioactivity adsorbed on the beads was counted in a LKB gamma counter.

To study immunoprecipitation of the unliganded receptor, the same solubilization and immunoprecipitation methods were used. After immunoprecipitation of the unliganded receptor, the quantities of receptors remaining in the supernatant were analyzed. The supernatants were incubated with 200 pmol/L [125I]ICYP for 30 minutes at 37°C, precipitated with 8% polyethylene glycol 6000, filtered and washed on glass filters (Whatman GF/F), and counted in a LKB gamma counter. Nonspecific binding was studied in the presence of 5 \( \mu \)mol/L propranolol.

Chronotropic Effects on Isolated Cardiac Myocytes

Cultured neonatal rat heart myocytes were used as a functional test system.17 Single cells were dissociated from the minced heart ventricle of 1- to 3-day-old Wistar rats with a 0.25% solution of trypsin. The myocytes were cultured as monolayers on the bottom of 45-mL Müller bottles (1.2×10⁶ seeded cells in 3 mL medium) in Halle SM20-1 medium18 containing 10% heat-inactivated calf serum and 2 \( \mu \)mol/L fluorodeoxyuridine, the latter to prevent proliferation of non-muscle cells. They were cultured for 4 days at 37°C in air.
The spontaneous beating frequency of myocytes was measured at 37°C on a heated stage of an inverted microscope. The changes in beating frequency were measured 5 minutes after the addition of the antibodies or the β-adrenergic agonist/antagonist. The basal beating rate was 160±20 beats per minute. The data represent each observation on 28 to 33 cells, or clusters, of synchronously contracting cells in three or four different cultures.

Cyclic AMP Determination on Rat Cardiomyocytes

Cyclic AMP (cAMP) accumulation on cultured rat heart myocytes was performed on 1 to 2×10⁶ cells in the presence of 500 μmol/L isobutylmethylxanthine as previously described using the protein-binding assay of Gilman. The experiments were repeated five times and are expressed as picomoles per milligram of protein.

Statistical Analysis

The increase in beating frequency compared with the corresponding control period before addition of antibody or isoproterenol was analyzed using Student’s paired t test. The data are given as mean±SEM. The immunoprecipitation data were analyzed with Student’s unpaired t test. A probability level of P<.05 was chosen as the least significant difference.

Results

Immunological Characterization of the Antibodies

Affinity-purified antibodies from patients with idiopathic DCM and from immunized rabbits were tested for the recognition of peptide sequence 197-222 corresponding to the second extracellular loop of the human β₁-adrenoceptor using an enzyme immunoassay. As shown in Fig 1, the rabbit antibodies against peptide 197-222 showed a much higher relative affinity for the peptide than did the patient autoantibodies.

Recognition of the β₁-adrenoceptor protein was tested by immunoprecipitation of solubilized [¹²⁵I]ICYP-receptor complex and of solubilized unliganded receptor. Autoantibodies from patients 5 through 7 and rabbit antibodies against peptide sequence 197-222 were unable to immunoprecipitate the antagonist-receptor complex. In contrast, rabbit antibodies against peptide sequence 34-57 immunoprecipitated the antagonist-receptor complex, confirming the adequacy of the test (Fig 2). All antibodies were able to immunoprecipitate the unliganded receptor, as shown by the decrease of ligand-binding sites in the supernatants of solubilized receptor after immunoprecipitation (Fig 3).

Fig 1. Plot of enzyme immunoassay on peptide 197-222 corresponding to the sequence of the second extracellular loop of the human β₁-adrenoceptor. Titration curves of affinity-purified autoantibodies are from pooled sera of four patients with idiopathic dilated cardiomyopathy (○-○) and of affinity-purified anti-peptide antibodies of rabbit (197-222)-1 (●-●), (197-222)-2 (△-△), and (197-222)-3 (□-□). Rabbits are identified as in Table 1. Values are expressed as absorbance at 405 nm.

Fig 2. Bar graph of immunoprecipitation of the solubilized antagonist-receptor complex from membranes of Escherichia coli expressing the human β₁-adrenoceptor. Results are expressed as the amount of [¹²⁵I]iodocyanopindolol (ICYP)-receptor complex in the precipitate (fmol±SD) from three experiments. Total amount of solubilized complex was 20 fmol. The results are shown for the IgG fraction of one rabbit before immunization (pre-immune), affinity-purified rabbit antibodies against the second extracellular loop [(197-222)-1, 2, 3], affinity-purified rabbit antibodies against the extracellular N-terminal region [(34-57)-2, 3], and affinity-purified autoantibodies from three patients with idiopathic dilated cardiomyopathy (patients 5 through 7).

Fig 3. Bar graph of immunoprecipitation of the solubilized unliganded receptor from membranes of Escherichia coli expressing the human β₁-adrenoceptor. Results are expressed as the amount of receptors determined at 200 pmol/L [¹²⁵I]iodocyanopindolol (ICYP) remaining in the supernatant after immunoprecipitation of the unliganded receptor (fmol±SD) from three experiments. The results are shown for the IgG fraction of one rabbit before immunization (pre-immune), affinity-purified rabbit antibodies against the second extracellular loop [(197-222)-1, 2, 3], affinity-purified rabbit antibodies against the extracellular N-terminal region [(34-57)-2, 3], and affinity-purified autoantibodies from three patients with idiopathic dilated cardiomyopathy (patients 5 through 7). All antibodies, with the exception of rabbit (34-57)-3, immunoprecipitated the unliganded receptor significantly (P<.05).
Chronotropic Response of Affinity-Purified Patient Antibodies and Rabbit Antipeptide Antibodies Against Two Different Sequences of the β1-Adrenoceptor

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration, nmol/L</th>
<th>Time of Incubation</th>
<th>Bisoprolol, 1 μmol/L</th>
<th>Peptide, 25 nmol/L</th>
<th>Isoproterenol, 10 μmol/L</th>
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<tr>
<td>Cardiomyopathy patients</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Patient pool (n=4)</td>
<td>0.25</td>
<td>33.0±2.8</td>
<td>32.4±4.9</td>
<td>0.8±2.0</td>
<td>0.8±1.6</td>
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<td>Patient 5</td>
<td>1.80</td>
<td>17.2±0.7</td>
<td>33.7±2.9</td>
<td>5.5±6.2</td>
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<tr>
<td>Patient 6</td>
<td>2.36</td>
<td>21.2±2.4</td>
<td>30.0±3.2</td>
<td>2.4±2.8</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 7</td>
<td>2.84</td>
<td>17.2±1.2</td>
<td>31.2±3.2</td>
<td>−0.8±2.8</td>
<td>ND</td>
</tr>
<tr>
<td>Flow through (patient 7)</td>
<td>−2.8</td>
<td>−11.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Immunized rabbits</td>
<td></td>
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<tr>
<td>Rabbit (197-222)-1</td>
<td>3.0</td>
<td>32.4±3.2</td>
<td>31.2±3.7</td>
<td>4.8±2.6</td>
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<tr>
<td>Rabbit (197-222)-2</td>
<td>2.0</td>
<td>14.8±1.4</td>
<td>19.6±1.5</td>
<td>2.7±1.2</td>
<td>0.66±1.0</td>
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<tr>
<td>Rabbit (197-222)-3</td>
<td>3.0</td>
<td>12.7±1.6</td>
<td>18.2±2.0</td>
<td>1.9±1.5</td>
<td>1.41±0.9</td>
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<tr>
<td>Rabbit (34-57)-2</td>
<td>3.0</td>
<td>1.7±1.3</td>
<td>4.9±2.3</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Rabbit (34-57)-3</td>
<td>3.0</td>
<td>1.1±0.9</td>
<td>1.1±1.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as means of increase in beats per minute (bpm) ±SE. ND indicates not determined.

*A truncated peptide (H16D) was used for neutralization.
†Peptide concentration was 12.3 μmol/L.

Functional Characterization of the Antibodies

The Table summarizes the chronotropic effect of autoantibodies from patients with idiopathic DCM and anti-peptide antibodies from immunized rabbits on cultured rat heart myocytes. Affinity-purified autoantibodies from pooled sera of four patients increased the beating frequency of cultured rat heart myocytes with a mean maximum of 32.4±4.9 beats per minute (P<.001). Individually purified autoantibodies from patients 5 through 7 increased the beating frequency to the same extent with mean maxima of 33.7±2.9, 30.3±3.2, and 31.2±3.2 beats per minute, respectively (P<.001). This stimulation corresponds to 65% to 80% of maximal isoproterenol stimulation. The bulk of the γ-globulin fraction from patient 7, which was not adsorbed on the immunosorbent, was completely devoid of stimulatory activity (flow through). Affinity-purified anti-peptide antibodies against the second extracellular loop from three different rabbits [(197-222)-1, 2, 3] showed the same stimulatory properties. They increased the beating frequency of cultured myocytes (P<.001), although they had different responses depending on the sera used for antibody purification.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Plot of dose dependency of the inhibition by bisoprolol of the positive chronotropic effect of the autoantibodies on rat cardiomyocytes. Bisoprolol was added at increasing concentrations to rat heart myocytes stimulated with affinity-purified autoantibodies from a patient with idiopathic dilated cardiomyopathy at 2 nmol/L. Increase in beating rate was 28±2.1 beats per minute. Mean ± SD, expressed as percent inhibition of increase in beating rate, is shown for seven observations per point.

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Plot of concentration dependency of the chronotropic effect induced by antibodies (Ab) directed against the second extracellular loop of the β1-adrenoceptor. The different concentrations of affinity-purified autoantibodies from pooled sera of four patients with idiopathic dilated cardiomyopathy (solid line) and affinity-purified anti-peptide antibodies of rabbit (197-222)-1 (open circle), (197-222)-2 (open triangle), and (197-222)-3 (open square) were cumulatively added to the medium of cultured neonatal rat heart myocytes, and the effects were tested after 5 minutes. A significant increase in beating frequency compared with basal rate (160±20 beats per minute) is expressed as mean ± SEM, corresponding to 25 to 30 observations (P<.05 to .001).
Control experiment in the absence of sera induced by antibodies to the beta-adrenergic receptor. Affinity-purified autoantibodies from pooled sera of four patients with idiopathic dilated cardiomyopathy (●—●) and anti-peptide antibodies from rabbit (197-222) were added to the medium of cultured neonatal rat heart myocytes at a concentration of 0.1 and 2.8 nmol/L, respectively. A control experiment using 10 μmol/L (-)-isoproterenol (●—●) was added. After 180 minutes, the medium was exchanged for fresh medium without antibodies. After 400 minutes, 1 μmol/L of the beta-blocking agent bisoprolol was added. A highly significant increase in beating frequency was observed at 5 and 60 minutes in the control experiment and at 5, 60, 180, 300, and 400 minutes in the antibody experiment (P<.001). At 65 and 90 minutes in the control experiment and at 420 minutes in the antibody experiments, the beating frequency was no longer significantly increased. Each value represents the mean±SEM from 10 to 25 observations.

The positive chronotropic effect was completely abolished by addition of the beta-selective adrenoceptor-blocking agent bisoprolol at a concentration of 1 μmol/L. In contrast, the beta-selective adrenoceptor antagonist ICI 118.551 at 0.1 μmol/L had no such effect (data not shown). A cumulative dose-response curve showed that the IC50 of bisoprolol on the stimulation by the autoantibodies was 0.1 μmol/L (Fig 4).

Furthermore, preincubation of both the autoantibodies and the anti-peptide antibodies with the antigenic peptide 197-222 at 25 nmol/L for 1 hour resulted in the antibodies no longer being able to induce stimulation. This confirms that the antibody effect is attributable to interaction of the antibody combining site with its target epitope on the myocytes and not to nonspecific effects induced by recognition of the antibody Fc fragment. In these studies, each experiment was followed by addition of isoproterenol at 10 μmol/L to confirm that the myocytes responded properly (Table).

To determine that only recognition of the second extracellular loop was responsible for the positive chronotropic effect induced by the antibodies, rabbit antibodies were induced and affinity purified against the extracellular N-terminal sequence 34-57 of the receptor. Although anti-peptide antibodies from the two rabbits [(34-57)-2, 3] were able to immunoprecipitate the solubilized [125I]ICYP-receptor complex (Fig 2), neither was able to induce a positive chronotropic effect (Table). This confirms that not all anti-beta-receptor antibodies can induce this effect simply by binding to the receptor.

The biochemical effects of the antibodies were tested by measuring the cAMP accumulation on treated rat heart myocytes. Although isoproterenol induced a 20-fold increase in basal cAMP accumulation (from 82.9±10.3 to 1648±12.3 pmol/mg protein), the antibodies had only a marginal although significant effect (from 139.5±8.6 to 167.5±7.6 pmol/mg protein). The cAMP accumulation in the presence of antibody and isoproterenol together was 1054±10.0 pmol/mg.

Concentration Dependency of the Functional Antibodies

The positive chronotropic effect of the autoantibodies from patients with idiopathic DCM was concentration dependent (Fig 5). This stimulation was significant at 0.02 nmol/L (P<.01) with a mean maximum at 0.41 nmol/L (P<.001). The anti-peptide antibodies from rabbit (197-222)-1, (197-222)-2, and (197-222)-3 also elevated the beating frequency of the isolated myocytes in a concentration-dependent manner. The threshold concentrations of these antibodies were 0.28 nmol/L (P<.001), 0.39 nmol/L (P<.01), and 0.58 nmol/L (P<.01), with maximal effects reached at concentrations of 5.69, 3.97, and 2.91 nmol/L, respectively. By comparing the titration of the antibodies in the enzyme immunoassay (Fig 1) with the concentration-response curve of the functional assay, it can be seen that autoantibodies of cardiomyopathic patients behave similarly in both assays with respect to their concentration, whereas the anti-peptide antibodies from rabbits are able to recognize the peptide with an apparent affinity 100 times higher than the concentration at which they are able to induce the positive chronotropic effect (Fig 5).

Time Dependency of the Functional Antibodies

In Fig 6, the stimulatory effect of the autoantibodies from patients with idiopathic DCM and anti-peptide antibodies of rabbit (197-222)-1 was investigated as a function of time. In sharp contrast to the chronotropic action of isoproterenol, which was subject to relatively rapid desensitization, the action of the antibodies was maintained at approximately peak levels for more than
6 hours. This action was not even reversed by washing and subsequent incubation for a few hours in fresh culture medium without the antibodies. The positive chronotropic effect was, however, completely and rapidly reversed by treatment with the \( \beta_1 \)-selective blocking agent bisoprolol at 1 \( \mu \text{mol/L} \).

When the agonist isoproterenol was added to autoantibody-stimulated myocytes, only a small increase in beating frequency was observed, and this increase was lower than that induced by the agonist alone. Moreover, in the presence of autoantibodies, the desensitization phenomenon normally induced by the agonist did not occur. When the autoantibodies and the agonist were washed out after the addition of 1 \( \mu \text{mol/L} \) bisoprolol, the cardiomyocytes responded to a new stimulation with isoproterenol as they did to a first stimulation. In contrast, isoproterenol restimulation of agonist-desensitized cells did not result in a significant increase in beating frequency (Fig 7).

**Discussion**

It has recently been suggested that autoantibodies against the \( \beta \)-adrenergic receptor were present in sera of patients with idiopathic DCM.\(^8\)-\(^12\) Similar results were obtained by our group using a different experimental approach to characterize autoantibodies. It was found that in about 30% of patients with idiopathic DCM, autoantibodies against the predicted second extracellular loop of the \( \beta_1 \)-adrenergic receptor were present.\(^9\) Because the second extracellular loop was found to be immunogenic in rabbits, this structure was investigated.\(^21\) This loop contains cysteine residues, which have been shown to interact with agonist binding and receptor activation.\(^22\),\(^23\) The autoantibodies from idiopathic DCM patients\(^9\) as well as the rabbit antibodies induced by immunization with a peptide corresponding to the sequence of the loop were able to inhibit radioligand binding on the receptor. This inhibition appeared to reflect a labile “activated” state of the receptor.\(^24\) On the other hand, it was found that the \( \gamma \)-globulin fraction of patients with DCM was able to induce a positive chronotropic effect on neonatal rat heart myocytes in culture.\(^20\),\(^21\) Thus, an effort was made to answer the question of whether the antibodies directed against the second extracellular loop of the human \( \beta_1 \)-adrenergceptor are identical to those that can induce a positive chronotropic effect. The immunological and functional data presented in this study give an unequivocally positive answer to that question.

The affinity-purified autoantibodies from patients with idiopathic DCM were stimulatory in the functional assay. Because the major part of the \( \gamma \)-globulin fraction, which was not adsorbed on the peptide immunosorbent, lacked any stimulatory effect, it was concluded that the stimulatory activity was attributable only to the recognition of the second extracellular loop of the receptor. A similar concentration dependency (0.02 to 0.41 \( \text{nmol/L} \)) was found both for epitopic recognition in an enzyme immunoassay and for the functional effect of the autoantibodies (Figs 1 and 5). These results contrast sharply with the action of affinity-purified rabbit anti-peptide (197-222) antibodies, which were able to recognize the peptide with an apparent affinity 1000 times higher than the concentration in which they were able to induce the positive chronotropic effect. This discrepancy can be explained by the fact that the antibody response in the patients is oligoclonal and directed completely toward the epitope on the peptide that is responsible for the physiological effect on the receptor, whereas the antibodies from the immunized rabbits are polyclonal in nature and directed against all the epitopes present on the second extracellular loop, with only a minor fraction directed at the epitope responsible for the physiological effect. It is even possible that the antibodies directed toward the nonfunctional epitopes may block the accessibility of the functional antibodies to their epitopic target through steric hindrance. This suggests the prevalence of protective antibodies over functional antibodies in the peptide (197-222)–induced immune response.

The epitope specificity of the stimulatory activity by the antibodies was further assessed by studying rabbit antibodies against an immunogenic region of the extracellular N-terminal sequence 34-57 of the human \( \beta_1 \)-adrenergceptor. These antibodies were able to immunoprecipitate the antagonist-receptor complex but were unable to increase the beating frequency of spontaneously beating rat heart myocytes in culture. In contrast, the antibodies against the second extracellular loop did not precipitate the antagonist-receptor complex but only the unliganded receptor. Thus, the functional effect of the autoantibodies is not attributable to the activation of the receptor by cross-linking with anti-receptor antibodies as is the case for immunological receptors\(^26\) or growth hormone receptors,\(^27\) but rather is caused by the interaction of the antibodies with a functional important epitope, which is not accessible on the antagonist-receptor complex.

Although the positive chronotropic effect of the antibodies cannot be washed out, addition of the \( \beta_1 \)-selective blocking agent bisoprolol to the myocytes results in complete inhibition of that effect. As shown elsewhere with active \( \gamma \)-globulin fractions from patients with cardiomyopathy, the \( \beta_1 \)-selective antagonist allows for the dissociation of the antibody-receptor complex because after antagonist treatment and addition of new medium to the myocytes, no stimulatory effect was observed.\(^12\)

The effects of the autoantibodies share some of the properties of a “partial agonist”: their effect remains submaximal even at saturating doses, with only a marginal effect on cAMP accumulation.\(^28\),\(^29\) Because it has been shown that a specific protein kinase for the \( \beta_1 \)-adrenergceptor does not exist\(^30\) and that desensitization of this receptor is only cAMP dependent, the nondesensitization by the autoantibodies could be explained by their marginal effect on cAMP accumulation. Nondesensitization by isoproterenol of receptors in the presence of autoantibodies could arise from the competition between the autoantibodies and the agonist. This hypothesis is in accordance with the observation that autoantibodies block cAMP accumulation induced by isoproterenol.\(^31\) Further experiments are needed to give a mechanistic interpretation to these results.

Autoantibodies against the second extracellular loop of the \( \beta_1 \)-adrenergceptor in patients with idiopathic DCM are able to induce an agonist-like stimulation of the cardiac myocytes. In contrast to classic adrenergic agonists such as isoproterenol, the autoantibodies fail to
induce desensitization of the receptor and then could lead or contribute to the maintenance of chronic sympathetic stimulation. This may explain the functional abnormalities in the absence of increased catecholamine levels. The prolonged overactivity may result in left ventricular hypertrophy and in subsequent heart failure as well as in lethal arrhythmias.32,33

The presence of autoantibodies with prolonged stimulatory β-receptor-mediated effects may, in some patients, be of pathophysiological importance. Because β1-blocking agents could break the strong binding to the β1-receptor of the antibody, this may explain to some extent the marked benefit of β1-blockade seen in some patients with idiopathic DCM.34-38 It is generally agreed that the sympathetic nervous system is activated in chronic heart failure to support the pump function of the failing myocardium.32,33 Prolonged sympathetic overactivity can, however, produce deleterious biochemical, metabolic, and electrophysiological effects in the heart, which may aggravate the heart disease.39 Sympathetic overstimulation will be counteracted by desensitization to adrenergic stimulation involving downregulation of the β-adrenergic receptors and their effector molecules (decrease in functional G proteins and adenylate cyclase, increase in functional G proteins).40-42 The large number of studies showing favorable long-term effects of β-blockers in patients with various forms of congestive heart failure give general support to the concept of a deleterious effect of long-term sympathetic overactivity.43 Chronic β-blockade has been found to not only prevent further damage but also improve heart function and, at least to some extent, normalize the β-adrenergic receptor–G protein–adenylate cyclase system.34,44

The nondesensitization observed with the autoantibodies appears to be in apparent contradiction with receptor downregulation observed in failing hearts. The observations made in vitro, however, are quite different from the situation in vivo, in which all complementatory immune components (eg, complement, cytotoxic cells, phagocytes) are present to induce immune endocytosis of the receptor. In the absence of any data linking directly the in vitro model to the in vivo model and in the absence of a mechanistic interpretation of the effect of the autoantibodies, no direct pathophysiological interpretation can be given to the observation presented here. We can, however, present a testable hypothesis. If the antibody-receptor complexes remain at the plasma membrane, the opportunity of immune downregulation can only increase. If, on the other hand, the β-blockers dissociate the antigen-antibody complex, they at the same time inhibit immune downregulation of the receptor and its target cell. The therapeutic efficacy of β1-selective β-blockers in some patients with DCM may be partly explained by their ability to dissociate the β-adrenergic receptor–antibody complex and to block continuous adrenergic overstimulation. Therefore, we might expect a more favorable response to long-term β-blockade in patients with circulating anti-receptor autoantibodies than in patients without such antibodies. A clinical study aimed at analyzing whether a correlation exists between the beneficial effects of β1-selective blocking agents and the presence of autoantibodies against the β1-adrenoceptor in patients with idiopathic DCM is in progress. If this correlation exists, the hypothesis forwarded here should be strengthened.

Acknowledgments

This work was supported by the Swedish Medical Research Council (projects 2529 and 9320); Swedish Heart Lung Foundation; Faculty of Medicine, University of Göteborg; and French Ministry of Research and Technology. The authors thank Prof A. Wollenberger for critical review of the manuscript. We thank Dr J.G. Guillet (Laboratoire d’Immunopharmacologie Moléculaire, Paris, France) for the gift of E. coli transfected with the gene of the human β1-adrenoceptor, Dr A. Vahine (Clinical Virology, Göteborg, Sweden) for peptide synthesis, Mr R. Mobini for his technical assistance, and Mr K. Sandell and Ms L. Schenck for help in preparing the manuscript.

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Autoimmunity in idiopathic dilated cardiomyopathy. Characterization of antibodies against the beta 1-adrenoceptor with positive chronotropic effect.
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_Circulation._ 1994;89:2760-2767
doi: 10.1161/01.CIR.89.6.2760

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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