Mapping of a Functional Autoimmune Epitope on the β_1-Adrenergic Receptor in Patients with Idiopathic Dilated Cardiomyopathy

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Abstract

The presence and properties of serum autoantibodies against β-adrenergic receptors in patients with idiopathic dilated cardiomyopathy were studied using synthetic peptides derived from the predicted sequences of the human β-adrenergic receptors.

Peptides corresponding to the sequences of the second extracellular loop of the human β_1- and β_2-adrenergic receptors were used as antigens in an enzyme immunoassay to screen sera from patients with dilated cardiomyopathy ($n = 42$), ischemic heart disease ($n = 17$), or healthy blood donors ($n = 34$). The sera of thirteen dilated cardiomyopathy patients, none of the ischemic heart disease patients, and four of the healthy controls monospecifically recognized the β_1-peptide. Only affinity-purified antibodies of these patients had a inhibitory effect on radioligand binding to the β_1 receptor of C6 rat glioma cells. They recognized the receptor protein by immunoblot and bound in situ to human myocardial tissue.

We conclude that a subgroup of patients with idiopathic dilated cardiomyopathy have in their sera autoantibodies specifically directed against the second extracellular loop of the β_1-adrenergic receptor. These antibodies could serve as a marker of an autoimmune response with physiological and/or pathological implications. (*J. Clin. Invest.* 1990. 86:1658–1663.) Key words: β-adrenergic receptor • dilated cardiomyopathy • auto-immunity • epitope mapping

Introduction

Autoantibodies against cell membrane receptors have been documented in a number of human diseases (1). Autoimmunity has been claimed as one of the pathologic processes involved in idiopathic dilated cardiomyopathy (2). Several findings support this hypothesis, such as the presence in patients' sera of autoantibodies directed against heart-specific antigens (3) or the imbalance between helper and cytotoxic T cells (4). Recently, autoantibodies against cardiac β-adrenergic receptors have been observed in patients with dilated cardiomyopathy (5). Clinical unresponsiveness to β_1-adrenergic stimulation could be explained by a marked decrease in the number of these receptors (6).

The primary sequences of the human β_1- and the human β_2-adrenergic receptors were recently derived from the corresponding DNA sequences (7–9). Starting from the predicted secondary structure of the receptors, we took a new approach to detect auto-antireceptor antibodies and to map the recognized epitopes by using peptides as synthetic antigens. In either receptor, the extracellular NH_2-terminal sequence does not seem to have a functional role in ligand binding or signal transduction. The only fragment involved in agonist binding affinity (10, 11), which contains both B- (12) and T-cell epitopes (13) and is accessible to antibodies, is the predicted second extracellular loop. Therefore, two peptides corresponding to the sequences of the predicted second extracellular loops of the human β_1- and β_2-adrenergic receptors were used as antigenic targets to detect receptor-specific antibodies. The sera of patients with idiopathic dilated cardiomyopathy were studied for the presence of autoantibodies directed against those hypothetical immunogenic regions.

Methods

Patient recruitment and evaluation. Patients with idiopathic dilated cardiomyopathy were selected from those admitted to the Department of Cardiology, Sahlgren's Hospital (Göteborg, Sweden) with diagnosis of heart failure and/or cardiomyopathy. The following pathological conditions were excluded from study: hypertrophic cardiomyopathy; previous myocardial infarction; coronary heart disease diagnosed by coronary angiography; severe hypertension; alcoholism; valvular heart disease; insulin-dependent diabetes mellitus; severe infection; cor pulmonale; mediastinal irradiation; and postchemotherapy cardiac dysfunction. All patients had echocardiographic findings consistent with dilated cardiomyopathy and left ventricular dysfunction with an ejection fraction below 45% in M mode tracing.

Sera of healthy patients were obtained from the blood donor bank of the hospital.

To evaluate the possible association between antireceptor antibodies and the myocardiopathic state, we included in the control group patients with ischemic heart disease. These patients had either a history of documented myocardial infarction or a coronary angiogram demonstrating multivessel disease, with at least one stenosis > 50% of the artery lumen diameter. They were selected on the basis of echocardiographic findings of dilated cardiomyopathy in the setting of their coronary artery disease.

Sera from 42 patients with idiopathic dilated cardiomyopathy were analyzed and compared with sera from healthy blood donors ($n = 34$) and patients with ischemic heart disease ($n = 17$). No β-blocking drugs were given to the patients for at least 3 wk before serum sampling. The clinical characterization of the patients is summarized in Table I.

Peptides. Peptides were synthesized by the solid phase method of Merrifield (14) using an automated peptide synthesizer (model no. 430A; Applied Biosystems, Inc., Foster City, CA). Peptides were desalted on a desalting grade molecular sieve (P6; Bio-Rad Laboratories, Cambridge, MA) using 0.1 M Na_2CO_3 as eluent and were stored in the

Table I. Clinical Profile of the Subjects under Investigation

Profile criteria	BD (n = 34)	IHD (n = 17)	DCM (n = 42)
Age	44±12	58±12	51±14
Sex F/M	11:33	2:15	8:34
Ejection fraction (%)	ND	31±10*	28±10*
Function class‡	1±0	3.3±0.6	2.7±1.0

The control group consisted of healthy blood donors (*BD*) and patients with ischemic heart disease (*IHD*). (*DCM*) Patients with idiopathic dilated cardiomyopathy. All values are expressed as mean±SD.
* In three patients with IHD and five patients with DCM, the ejection fraction could not be determined from the echocardiographic data.
‡ Function class according to the New York Heart Association.

same solvent at −20°C until use. Composition of the purified peptides was verified in an automated aminoacid analyzer (Beckman Instruments, Inc., Palo Alto, CA). The peptides correspond to the hypothesized second extracellular loop of the β-receptors. The β_1-peptide corresponds to the sequence of amino acids 183–208 of the human β_1-adrenergic receptor (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) (9), and the β_2-peptide to the sequence 172–197 of the human β_2-adrenergic receptor (H-W-Y-R-A-T-H-Q-E-A-I-N-C-Y-A-N-E-T-C-C-D-F-F-T-N-Q) (7, 8). Both peptides have a cysteine group as carboxy terminus.

Preparation of antibodies. Whole sera or affinity chromatography–purified antibodies were used in the different experiments. Immunoglobulin fractions were prepared from sera by precipitation in 50% $(NH_4)_2SO_4$. The precipitate was redissolved in phosphate (20 mM) buffered saline (pH 7.4) in half of the initial volume and dialyzed twice against the same buffer. For affinity purification, pooled immunoglobulin fractions of three positive sera from healthy controls and three positive sera from patients with dilated idiopathic cardiomyopathy were loaded on a Sepharose 4B CNBr-activated substrate to which the β_1-peptide was covalently linked (15). After washing of the immunosorbent with PBS, the specific anti–β_1-peptide antibodies were eluted with 0.2 M glycine (pH 2.8), neutralized in 1 M Tris (pH 8.0), and extensively dialyzed against PBS.

As a positive control, rabbit monospecific affinity-purified antibodies against the β_1-peptide, characterized elsewhere (15), were used.

Enzyme immunoassay. 50 μl of a 0.1 M Na_2CO_3 solution supplemented with 1% (vol/vol) β-mercaptoethanol containing 50 μg/ml of peptide was adsorbed for 1 h at room temperature on NUNC (Kanstrup, Denmark) microtiter plates. The wells were then saturated with PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) supplemented with 3% (wt/vol) of skimmed milk, 0.1% (vol/vol) of Tween 20 (E. Merck, Darmstadt, FRG), and 0.01% (wt/vol) of merthiolate (Sigma Chemical Co., St. Louis, MO) (PMT).[1] 50 μl of dilutions of the sera from 1:20 to 1:160 in PMT were allowed to react with the peptides overnight at 4°C. After washing the wells three times with PMT, 0.05 ml of an affinity-purified biotinylated rabbit anti–human IgG antibody solution diluted 1:1,000 in PMT was allowed to react for 1 h at room temperature. After three more washings, the bound biotinylated antibody was then detected by incubation of the plates for 1 h at room temperature with 0.05 ml/well of a 1 μg/ml solution of streptavidin-peroxidase (Sigma Chemical Co.) in PMT followed by three washing in PBS and addition of the chromogenic substrate H_2O_2 (2.5 mM)-2,2'-azino-di-(ethyl-

benzthiazoline) sulfonic acid (2 mM) (ABTS, Sigma Chemical Co.). After 30 min, optical densities were read at 405 nm in a TITERTEK ELISA-reader (Flow Laboratories, Irvine, Scotland).

Western blots of human β_1-adrenergic receptors. To avoid cross-reactivity between β-adrenergic receptor subtypes and to increase the sensitivity of immunoblots, we used as antigen membrane preparations of *Escherichia coli* expressing human β_1-adrenergic receptors. We already showed that *E. coli*, transformed with the appropriate vectors express human β-adrenergic receptors that retain their binding properties and are detectable by immunoblots (16). The gene coding for the human β_1-adrenergic receptor was fused in phase to the 3' terminus of the MalE gene that codes for a bacterial periplasmic maltose binding protein (17). Spheroplasts from *E. coli* expressing the hybrid malE human β_1-adrenergic receptor were prepared as described (18) and lysed in 10 mM Tris, 1 mM EDTA buffer (pH 7.4). Membranes were recovered by centrifugation at 100,000 g and resuspended in the same buffer to which a cocktail of protease inhibitors was added (19). The membrane proteins were subjected to electrophoresis on a 10% polyacrylamide gel in SDS according to Laemmli (20), subjected to electrotransfer to nitrocellulose (21), and the latter was saturated for 2 h with PMT. Affinity-purified antibodies from positive sera of three patients with dilated idiopathic cardiomyopathy were diluted in PMT to a titer comparable with a dilution of 1:20 of whole sera and were incubated at 4°C overnight with the β_1-adrenergic receptor blots. After washing in PMT, the nitrocellulose strips were incubated with a 1:500 dilution of affinity-purified biotinylated rabbit anti–human IgG for 1 h at room temperature and subsequently with 1 μg/ml a streptavidin-peroxydase solution in the same buffer. Strips were washed in PMT and in PBS containing 0.1% Triton X-100 (E. Merck) before adding the chromogenic substrate H_2O_2-4-chloro-1-naphtol (Sigma Chemical Co.). As soon as protein bands were revealed, strips were washed extensively in water and stored at −20°C. Profiles of the immunoblots were compared to those obtained with affinity-purified rabbit anti–human β_1-adrenergic receptor antibodies as previously described (15).

Immunohistochemistry on human myocardium. Endomyocardial biopsies from normal human donor hearts taken before reperfusion at cardiac transplantation, formalin fixed and paraffin embedded, were studied to visualize recognition of the human β_1-receptor. An avidin-biotin method was used. To block endogenous peroxidase activity all sections were pretreated with 0.5% H_2O_2 in methanol after deparaffination in xylene and digestion at 37°C for 10 min with 0.05% trypsin (Sigma Chemical Co.). Endogenous human IgG stained strongly positive in both control and antibody-incubated sections, but preincubation with rabbit anti–human IgG antibodies at a dilution of 1:1,600 efficiently blocked this activity. Affinity-purified antibodies prepared from positive sera of three patients with dilated idiopathic cardiomyopathy were used as primary antibodies at a concentration of 10 μg/ml and incubated with tissue sections for 18 h at 4°C. The sections were next incubated with biotinylated rabbit anti–human IgG at a dilution of 1:400, and, finally, the sections were incubated with a streptavidin-peroxidase complex. To visualize the immune complexes, the sections were treated with 0.05% diaminobenzidine hydrochloride with 0.02% H_2O_2 in TBS (pH 7.4) for 2 min. Nuclear staining was performed with Mayer's solution at a dilution of 1:2 for 3 min. Sections treated as the controls with the omission of incubation with the affinity-purified antibodies were used as negative controls.

Radioligand binding. Membranes of C6 rat glioma cells, rich in β_1-adrenergic receptors (22), were prepared by homogenization of the cells in 25 mM Tris (pH 7.4) containing 10% sucrose. After centrifugation at low speed to discard the debris, membranes were ultracentrifuged at 100,000 g for 60 min. The pellet was resuspended in 25 mM Tris (pH 7.4), 75 mM $MgCl_2$ to which up to 10% glycerol was added. Membrane protein concentration of the suspension was 8 mg/ml. 100 μl of the membrane suspension were incubated overnight at 4°C with the same volume of affinity-purified antibodies at 62 μg/ml. Controls were set up by incubation under similar conditions in the presence of human IgG. Saturation binding experiments were performed by incubation of 100 μl of the membrane–antibody mixture after dilution to

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1. *Abbreviation used in this paper:* PMT, PBS (10 mM phosphate, 140 mM NaCl, pH 7.4) supplemented with 3% (wt/vol) of skimmed milk, 0.1% (vol/vol) of TWEEN 20 (E. Merck, Darmstadt, FRG), and 0.01% (wt/vol) of merthiolate.

2.5 ml in a 25 mM Tris (pH 7.4), 75 mM $MgCl_2$ solution supplemented with 1 mg/ml ascorbic acid with increasing amounts of ^{125}I-(−)-iodo-cyanopindolol (200 Cu/mmol; Amersham International, UK) (final volume 200 μl) at 37°C for 60 min before filtration on GF/F glass filters (Whatman Inc., Clifton, NJ), washing with cold buffer, and counting radioactivity in a gamma-counter (LKB Instruments, Inc., Gaithersburg, MD). Blanks were set up in the presence of 2.5 μM of the unlabeled antagonist dl-propranolol. Competition binding experiments with the agonist (−)-isoproterenol (Sigma Chemical Co.) were performed under the same conditions at 100 pM of the radioligand but with increasing amounts of the competitor. Finally, a dose response study was set up by preincubating overnight at 4°C a membrane suspension with increasing amounts of antibody in a final volume of 500 μl before performing the saturation binding experiments with ^{125}I-(−)-iodocyanopindolol. Saturation binding curves were analyzed by the nonlinear regression method of Wilkinson (23).

Results

Detection of autoantibodies. When positivity was defined as 2.5 times the background optical density, thirteen sera of patients with dilated cardiomyopathy (31%) and four sera of the healthy control group (12%) monospecifically recognized the $β_1$-peptide at dilutions varying from 1:20 to 1:160 (Fig. 1). The number of sera positive for both peptides was 2:42 in the idiopathic dilated cardiomyopathy group, 2:17 in the ischemic group, and none in healthy controls. The difference between the healthy control group or the group of ischaemic patients and the patients with dilated idiopathic cardiomyopathy was significant at the 95% level as determined by an ANOVA analysis (Statview). No sera only recognized the $β_2$-peptide. Two to three repetitions of the enzyme immunoassay yielded consistent results and subsequent blood samples of positive patients
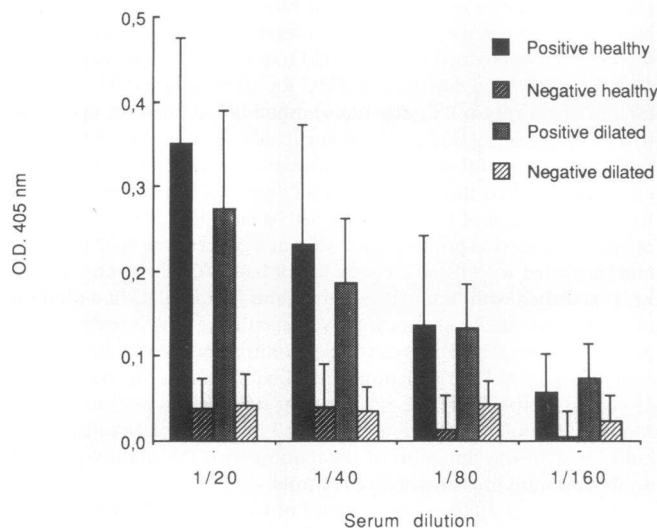
showed the persistence of the antibodies over a period of up to 15 wk, whereas over the same period no initially negative serum became positive. The sera of two patients and two positive controls were shown to remain positive 1 yr after the first analysis.

Characterization of autoantibodies. Affinity-purified antibodies of positive control sera (a pool of three positive sera from healthy controls) and patients (a pool of three positive sera from patients with idiopathic dilated cardiomyopathy) were tested on the $β_1$-peptide. Antibodies from sera of positive healthy controls had a higher avidity for the peptide compared to those of the patients (Fig. 4). To confirm that the positive anti-$β_1$-peptide response in EIA was a marker for the recognition of the $β_1$-adrenergic receptor, Western blots were developed. Human $β_1$-adrenergic receptor, expressed as a fusion protein in *E. coli* transfected with the human receptor gene, was used as target for the affinity-purified autoantibodies. Autoantibodies stained three proteins of molecular masses 64, 59, and 51 kD that were specific for the $β_1$-adrenergic receptor as shown by inhibition of staining of these bands after preincubation with the $β_1$-peptide (Fig. 2). This pattern corresponds to the partially degraded fusion protein as shown by their specific recognition with affinity purified rabbit antibodies raised against the $β_1$-peptide (15).

The affinity-purified human $β_1$-peptide antibodies prepared from positive sera of patients were also studied by immunohistochemistry to investigate the ability to recognize the $β_1$-adrenergic receptor in human myocardium. Sections incubated with the auto-antihuman $β_1$-receptor antibodies showed positive reactions in vessel walls and the sarcolemma of cardiac myocytes (Fig. 3).

Finally, the functional relevance of the affinity-purified antibodies was studied by ligand binding studies on C6 rat glioma cell membranes carrying ~ 80% of $β_1$- and 20% of $β_2$-adrenergic receptors (22). As shown in Fig. 4 *B*, preincubation with the patient antibodies resulted in a decrease of the number of binding sites without change in the dissociation constant suggesting a noncompetitive inhibition. Antibodies purified from positive sera of healthy controls did not show this effect. The remaining binding sites did not show any change in the affinity for the agonist (−)-isoproterenol (Fig. 4 *C*). Finally, a dose response study showed that a maximal response (~ 70% of inhibition) was obtained for concentrations ranging from 330 nM to 21 nM IgG and disappeared under 4.1 nM of antibody.
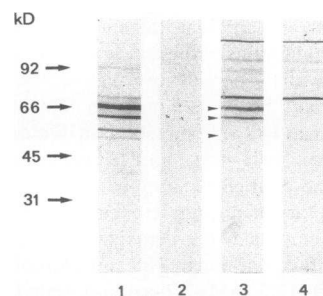
Figure 1. Enzyme immunoassay on the $β_1$-peptide with sera from healthy blood donors and from patients with dilated cardiomyopathy. The mean and SD of the optical density at 405 nm are give for four serum dilutions. Sera from healthy blood donors (*n* = 34) were divided in positive sera (4:34) and negative sera (30:34); sera from patients with dilated idiopathic cardiomyopathy (*n* = 42) were divided in positive sera (13:42) and negative sera (29:42). The difference between negative and positive sera was highly significant (P < 0.001) for the dilutions at 1:20, 1:40, and 1:80 and significant (P < 0.01) for the dilution at 1:160 (Student's test).

Figure 2. Western blots on the membrane proteins of *E. coli* expressing the human $β_1$-adrenergic receptor. (Lane *1*) Proteins revealed with affinity-purified rabbit anti-$β_1$-peptide antibodies (15). (Lane *2*) Affinity-purified rabbit anti-$β_1$-peptide antibodies preincubated with the peptide before incubation with the blotted proteins. (Lane *3*) Proteins revealed with affinity-purified human autoantibodies. (Lane *4*) Affinity-purified human autoantibodies preincubated with the peptide before incubation with the blotted proteins. Three protein bands (*arrows*), corresponding to degradation products of the fusion protein, are specifically stained with both antibodies.
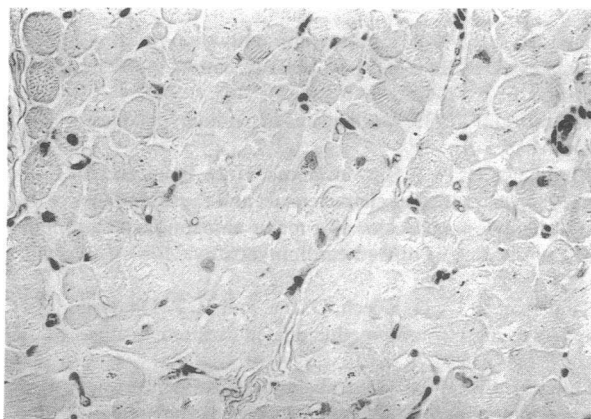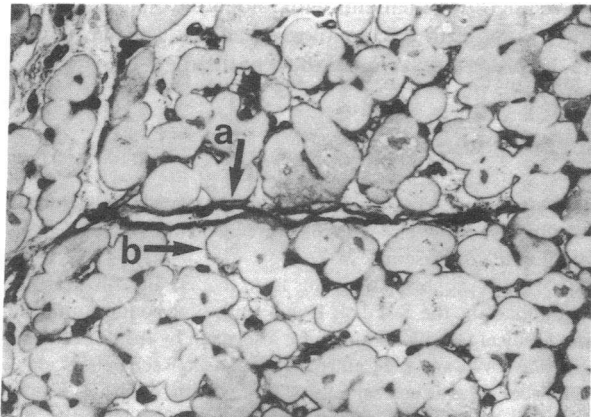
Figure 3. Immunohistochemistry of myocardiac tissue with affinity-purified autoantibodies. Normal human myocardium developed with affinity-purified human autoantibodies from a pool of three positive patient's sera as described under Methods. (*Top*) There is a strong staining of the vessels (*a*), but also a well-defined positive reaction in the sarcolemma of myocytes (*b*). The same staining pattern was observed using rabbit antipeptide antibodies on rabbit myocardium (15). (*Bottom*) Negative control including all steps needed for positive staining with the exception of incubation with the human affinity-purified autoantibodies.

These results allowed an estimation of the apparent avidity of the antibodies for the receptor at ~ 10 nM.

Discussion

In this study, we report evidence for the presence of autoantibodies against the β_1-adrenergic receptor in sera from a subgroup of patients with idiopathic dilated cardiomyopathy. We have localized the domain of recognition to the second extracellular loop of the β_1-adrenergic receptor.

Recently, it was suggested that antibodies against the β-receptor were present in sera of patients with dilated cardiomyopathy (5). This was shown by the ability of sera from such patients to inhibit binding of radiolabeled antagonist to rat cardiac membranes and to immunoprecipitate solubilized receptors. The methodology was not discriminative for a receptor subtype. It was, however, shown that anti-HLA alloimmune antibodies could also immunoprecipitate β-adrenergic
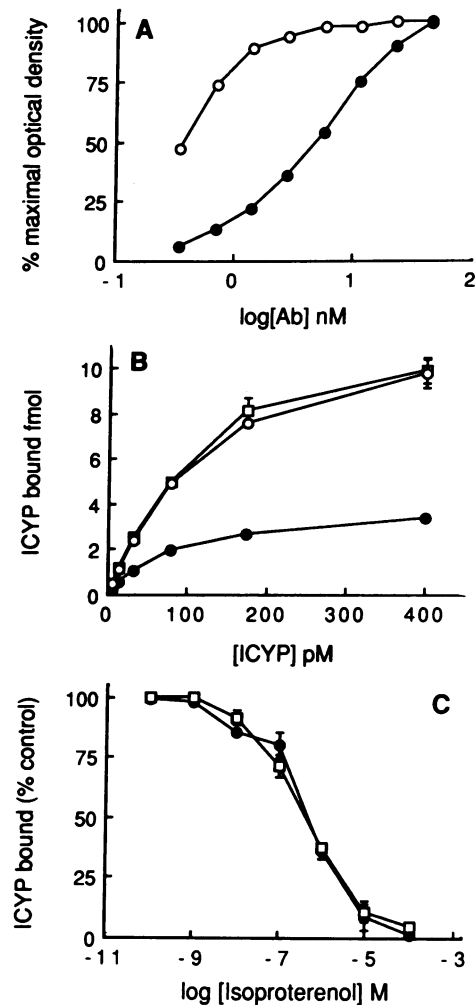

Figure 4. Properties of affinity-purified antibodies from positive sera of healthy controls and positive sera of patients with dilated idiopathic cardiomyopathy. (*A*) Enzyme immunoassay on the β_1-peptide with the affinity-purified antibodies from a pool of three positive sera from healthy controls (o) and from a pool of three positive sera from patients (●). The maximal response was determined as the OD value at 405 nm (OD = 1.06) for the highest concentration of the positive control antibodies. (*B*) Binding isotherms of $^{125}I\text{-}(-)$-iodocyanopindolol binding to membranes of rat C6 glioma cells preincubated overnight at 4°C in the presence of control human IgG (□) or affinity-purified autoantibodies from positive sera of healthy controls (o) and from sera of patients (●). The mean and standard deviations of three binding experiments are shown. (*C*) Competition curves of the agonist $(-)$-isoproterenol for $^{125}I\text{-}(-)$-iodocyanopindolol binding to membranes of rat C6 glioma cells preincubated overnight at 4°C in the presence of control human IgG (□) or affinity-purified autoantibodies from sera of patients (●). No significant differences could be shown. The mean and standard deviations of four experiments are shown.

receptors and inhibit radioligand binding on these receptors (24). Both criteria are thus inconclusive for the presence of auto-antireceptor antibodies.

To avoid these pitfalls, we sought a different experimental approach to characterize auto-antireceptor antibodies in cardiac patients. Based on the putative structure of the human β-adrenergic receptors, we predicted a sequence that might be involved in an autoimmune recognition of the β-adrenergic

receptor. Three criteria were used to justify the selection of this sequence. First, the sequence should be accessible at the extracellular side of the receptor-bearing cell as is the case for the major immunogenic region against which auto-antinicotinic receptor antibodies are directed in myasthenia gravis (25). Second, the sequence should include B-cell epitopes to be antigenic. The effective antigenicity of the selected sequence was confirmed by raising antibodies against the corresponding free peptide in rabbits (15). A third, less stringent criterion, was the potential functional importance for ligand binding of the selected sequence (10, 11).

The results presented here effectively show the existence of autoantibodies directed against the selected amino acid sequence. Most of the positive sera were specific for the sequence of the human β_1-adrenergic receptor. This indicates the existence of subtype-specific epitopes despite the overall homology ($\sim$ 60%) between the amino acid sequences of the β_1-peptide and the β_2-peptide.

The purified anti-β_1-peptide antibodies of patients recognize the β_1-adrenergic receptor as shown by immunoblots on membrane proteins of *E. coli* transfected with the receptor gene. The successful staining of myocardial tissue sections with these auto-antibodies indicated that they also bind to the membrane receptor. These results taken together suggested that peptide recognition was due to autoantibodies against the β_1-adrenergic receptor.

Ligand binding studies of the affinity-purified auto-antibodies on the β_1-adrenergic receptor showed the ability to decrease in vitro the number of radioligand binding sites without significantly changing the affinity for antagonist or agonist. While affinity-purified antibodies from control sera displayed higher affinity for the peptide, they had no such inhibitory effect; this suggests that they are directed against nonfunctional epitopes on the sequence. The autoantibody selectivity for the β_1-adrenergic receptor was further assessed in our experiments by the fact that the maximal decrease of binding sites on C6 cell membranes ($\sim$ 75%) corresponds to the percentage of β_1-receptors on those cells that also carry up to 20% of β_2-receptors. The functional effect of the autoantibodies is consistent with the selective β_1-receptor downregulation reported on failing human ventricular myocardium (26). Comparison between the titers of the affinity-purified antibodies and of those of the sera by an enzyme immunoassay on the β_1-peptide (data not shown), show that the concentration of antibodies in the serum exceeded at least 10 times the avidity constant; the concentration of autoantibodies in the serum is therefore sufficient to inactivate β_1-adrenergic receptors in vivo. The low amount and the polyclonality of the purified antibodies did not allow a mechanistic approach of the inactivation process. Human or murine monoclonal antibodies displaying the same properties as these autoantibodies will be needed to elucidate this question.

The high incidence (12%) of antipeptide antibodies in an apparently healthy population might be due to cross-reactivity with an ubiquitous microbial antigen (27). The immune response may vary with the B cell repertoire of each individual. Only in a minor population could recognition of a specific epitope lead to induction of inhibitory autoantibodies and thus to functional interference with the β_1-adrenergic receptors.

In conclusion, we have identified a functionally important epitope on the β_1-adrenergic receptor, recognized by autoantibodies in a subgroup of patients with idiopathic dilated cardiomyopathy. Long term epidemiological studies are needed to evaluate the prognostic value of these antibodies.

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