Journal of the American College of Cardiology
© 2000 by the American College of Cardiology
Published by Elsevier Science Inc.

Vol. 36, No. 4, 2000
ISSN 0735-1097/00/$20.00
PII S0735-1097(00)00881-0

Modulation of Beta$_1$-Adrenoceptor Activity by Domain-Specific Antibodies and Heart Failure–Associated Autoantibodies

Roland Jahns, MD,*† Valérie Boivin, PHD,† Thorsten Krapf, MD,† Gerd Wallukat, MD,‡ Fritz Boege, MD,* Martin J. Lohse, MD†

Wuerzburg and Berlin, Germany

OBJECTIVES Our study attempted to gain further understanding of the allosteric effects of human autoantibodies on beta$_1$-adrenergic receptor (beta$_1$-AR) function.

BACKGROUND Recently, we reported on the existence of activating anti-beta$_1$-AR antibodies in patients with dilated cardiomyopathy (DCM 26% prevalence) or ischemic cardiomyopathy (ICM, 10% prevalence); however, their functional effects have not yet been thoroughly characterized.

METHODS In this study we detected functionally active receptor-antibodies in 8 out of 30 DCM patients. Their immunological and functional properties were analyzed using both synthetic receptor-peptides and intact recombinant human beta$_1$-AR, and were compared with those of heterologous antibodies to selected beta$_1$-AR domains generated in rabbits and mice.

RESULTS Rabbit, mouse, and human anti-beta$_1$-AR against the second extracellular domain preferentially bound to a native receptor conformation and impaired radioligand binding to the receptor. However, their functional effects differed considerably: Rabbit and mouse antibodies decreased both basal and agonist-stimulated cAMP production, whereas the patient antibodies (n = 8) increased basal, and six of them also increased agonist-stimulated receptor activity (i.e., acted as receptor-sensitizing agents). Two out of eight human anti-beta$_1$-AR increased basal but decreased agonist-stimulated receptor activity (i.e., acted as partial agonists).

CONCLUSIONS Antibodies against the same small beta$_1$-AR domain can have very divergent allosteric effects, ranging from inhibitory to agonist-promoting activities. Activating autoantibodies were associated with severe cardiac dysfunction and thus might be involved in the development and/or course of human cardiomyopathy. (J Am Coll Cardiol 2000;36:1280–7) © 2000 by the American College of Cardiology

A majority of hormones and neurotransmitters exert their physiological effects through G protein–coupled receptors. In addition, antibodies specifically directed against certain members of this superfamily have been shown to be able to affect receptor-mediated cellular signaling as well (1). Some of these antibodies are supposed to be involved in the initiation and/or course of several human autoimmune diseases: Receptor-modulating autoantibodies have been found against the thyrotropin receptor, nicotinic acetylcholine receptor, and the insulin receptor, suggesting an implication in the pathogenesis of Graves' disease (2), myasthenia gravis (3), and type B insulin-resistant diabetes (4), respectively. More recently, autoantibodies targeting the human beta$_1$-adrenergic receptor (beta$_1$-AR) have been detected in a substantial fraction of patients with idiopathic dilated cardiomyopathy (DCM) (5–7) and, with lower frequency, also in patients with ischemic cardiomyopathy (ICM) (7). Because of the use of different strategies for the detection and characterization of such anti-beta$_1$-AR autoantibodies the reports on their prevalence, their functional properties, and their (supposed) pathophysiological role are not yet conclusive.

In this study we used an approach based on several newly developed recombinant tools (7–10) to investigate whether and to what extent anti-beta$_1$-AR might interfere with receptor-signaling: As a first step, we utilized fusion proteins with selected domains of the human beta$_1$-AR (11) to generate *heterologous* anti-beta$_1$-AR in rabbits (polyclonal) and mice (monoclonal). We then utilized recombinant human beta$_1$-AR expressed in various cells to characterize their immunological and functional properties (7,8,12). Finally, these properties were compared with those of selected human anti-beta$_1$-AR autoantibodies, representative for a certain subgroup of cardiomyopathic patients (7,13).

METHODS

Generation of heterologous antibodies against the human beta$_1$-adrenergic receptor. Fusion proteins of bacterial glutathion-*S*-transferase (GST) and the aminoterminus

From the *Department of Internal Medicine, Medizinische Poliklinik, University of Wuerzburg, Wuerzburg, Germany; †Institute of Pharmacology, University of Wuerzburg, Wuerzburg, Germany; and ‡Max Delbrück Center for Molecular Medicine, Berlin, Germany. The study was supported by the Deutsche Forschungsgemeinschaft, Ja 706/2-1. R. Jahns and V. Boivin contributed equally to this work.

Manuscript received December 13, 1999; revised manuscript received April 10, 2000, accepted June 13, 2000.

Abbreviations and Acronyms

anti-beta$_1$-AR/-ECII	= antibodies against the beta$_1$-adrenergic receptor/second extracellular loop of the beta$_1$-adrenergic receptor
beta$_1$-AR	= beta$_1$-adrenergic receptor
beta-Me	= beta-mercaptoethanol
BSA	= bovine serum albumin
DCM	= dilated cardiomyopathy
ELISA	= enzyme-linked immunoassay
GST	= glutathion-*S*-transferase
IBMX	= isobutylmethylxanthine
PBS	= phosphate buffered saline
SDS	= sodium dodecyl sulfate

(N), carboxyterminus (C), or second extracellular domain (ECII) of the human beta$_1$ (11) and beta$_2$-AR (14) were produced in *Escherichia coli* as previously described (8). To generate anti-beta$_1$-AR in rabbits and mice, 50 to 100 μg of the purified beta$_1$-AR fusion proteins in incomplete Freund's adjuvant were administered subcutaneously (New Zealand White rabbits) or intraperitoneally (Balb/c mice). Subsequent boosts were given at four-week intervals; mice received an additional antigen boost intravenously three days prior to fusion. Sera were drawn 6 and 12 weeks after immunization and assayed for reactivity with the corresponding antigens by enzyme-linked immunoassay (ELISA). The IgG was prepared from immunoreactive rabbits by caprylic acid precipitation and immunopurified using sodium dodecyl sulfate (SDS)-denatured recombinant human beta$_1$-AR expressed in Sf9 cells (8). For monoclonal antibodies, the spleens from immunoreactive mice were removed and fused with nonsecreting Ag8 myeloma cells (15) as described elsewhere (16). Three weeks after fusion the supernatants of the hybridomas were assayed for immunoreactivity by ELISA; positive clones were subcloned by the limiting dilution technique (17), and subtype and concentration of the produced immunoglobulins were determined by a commercially available kit (Boehringer Mannheim, Germany).

Preparation of human IgG fractions. Thirty patients suffering from idiopathic dilated cardiomyopathy (DCM) were included in the present study (6 women and 24 men; mean age: 55 ± 9 years; NYHA functional class III–IV; left ventricular diastolic volume >110 ml/m^2; see Table 1). According to the recently defined criteria (7), DCM was diagnosed when coronary heart disease was excluded by angiography, and when exposure to cardiotoxic substances, myocarditis, or other systemic heart diseases were not evident from clinical history. All patients were stable under therapy with angiotensin-converting enzyme (ACE)-inhibitors, diuretics, nitrates, and digitalis. None of them received beta-receptor agonists or beta-blockers. The IgG was prepared from the sera either 1) by direct caprylic acid precipitation (n = 20) or 2) by nonselective immunoadsorbtion and elution from protein G columns as previously described (13), also followed by caprylic acid precipitation (n = 10). Prior to the assays all samples were extensively dialyzed against phosphate buffered saline (PBS) and normalized to 1,000 μg IgG/ml (7). Control IgG was obtained from 15 healthy subjects with normal left ventricular function (4 women and 11 men; mean age 49 ± 11 years; see Table 1).

Enzyme-linked immunoassay. Supernatants of B cell hybridomas (mouse spleen-/Ag8-cells), immunopurified rabbit antibodies, and human IgG-preparations were assayed for immunoreactivity first by a conventional ELISA: GST was cleaved off from the fusion proteins (see above) by thrombin, and fragments corresponding to selected domains of the human beta$_1$- or beta$_2$-AR (N-terminus, C-terminus, and ECII-domain, respectively) were coated onto 96-well plates (5 ng protein/well). Bovine serum albumin (BSA) and a nonreceptor peptide served as specificity controls (7). The antigens were either reduced or not with 1% beta-mercaptoethanol (beta-Me) and incubated with serially diluted antibodies for 2 h at 37°C (rabbit anti-beta$_1$-N/-C/-

Table 1. Clinical and Hemodynamic Data of Patients and Healthy Subjects†

Parameter	DCM (+) Subgroup A (n = 6)	DCM (+) Subgroup B (n = 2)	DCM (−) (n = 22)	Healthy Subjects (n = 15)
General				
Age (yrs)	56 ± 5	53 ± 9	55 ± 8	49 ± 11
Gender: female/male	2/4	0/2	4/18	4/11
NHYA functional class	3.2 ± 0.3*	3.1 ± 0.4*	2.6 ± 0.5	0
Heart rate (min^{-1})	89 ± 3*	88 ± 5*	84 ± 6	69 ± 18
LV Angiography				
SVR (dyn·s·cm^{-5})	1,404 ± 104	1,410 ± 18	1,420 ± 115	n.d.
LVEF (%)	29.2 ± 3.2**	28.5 ± 2.1**	37.2 ± 5.2	n.d.
CI (l·min^{-1}·m^{-2})	2.26 ± 0.4*	2.20 ± 0.3*	2.79 ± 0.5	n.d.
Cont. (mm Hg·s^{-1})	752 ± 87*	729 ± 38*	858 ± 113	n.d.

†Mean values ± SD. Patients with dilated cardiomyopathy (DCM), who were positive (+) or negative (−) for functionally active anti-beta$_1$-ECII were compared by the Student *t*-test for unpaired samples. Significant differences between DCM (+) and DCM (−) are denoted by *$p < 0.05$ or **$p < 0.005$.

SVR = systemic vascular resistance; CI = cardiac index; LVEF = left ventricular ejection fraction (ventriculography); Cont. = contractility (peak dP/dt), derived from left ventricular high fidelity time/pressure curves; n.d. = not determined.

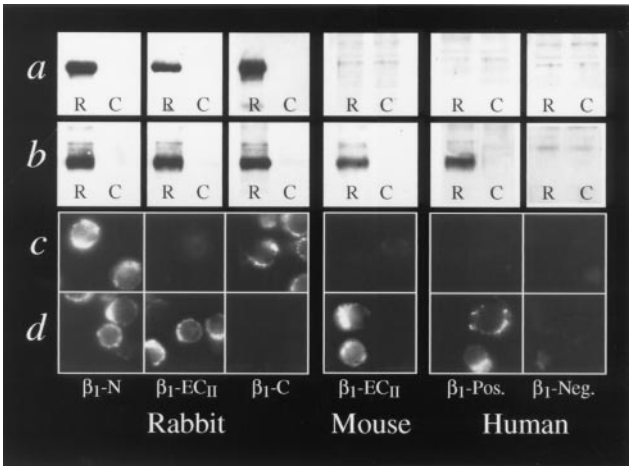

Figure 1. Detection of anti-beta$_1$-AR by various approaches. **(a)** Western blots of crude cell lysates, and **(b)** immunoprecipitates of membrane-biotinylated Sf9 cells expressing recombinant human beta-1-AR **(R)** or the wildtype vector **(C)**, and **(c)** fixed and permeabilized, or **(d)** intact unfixed Sf9-beta$_1$ cells were probed with either (a, c, d) domain-specific rabbit anti-beta$_1$-N/-ECII/-C (Rabbit) or mouse anti-beta$_1$-ECII (Mouse), or (b) with POD-coupled streptavidin (see Methods). Their respective immunoreactivities are compared with those of anti-beta$_1$-ECII-positive or -negative cardiomyopathic patients (Human, Pat. β_1-Pos./-Neg.). Note, that SDS-denatured beta$_1$-AR **(lane a)** are stained less clearly (Rabbit) or not at all (Mouse, Human) by anti-beta$_1$-ECII antibodies, whereas all of them immunoprecipitate **(lane b)** or recognize the native beta$_1$-AR **(lane d)**.

ECII: 50 to 32,000-fold; mouse IgM: 50- to 1,280-fold; human IgG: 5 to 500-fold). Bound antibodies were detected with appropriate species-specific horseradish peroxidase conjugated secondary antibodies and *o*-phenylene-diamine (Sigma, Deisenhofen, Germany) as substrate. Optical densities were read at 490 nm.

Western-blotting and immunoprecipitation. The SDS-lysates (2% SDS, 1% beta-Me, 60 mmol/liter Tris-HCl, pH 6.4) of Sf9 cells infected either with recombinant *Autographa californica* nuclear polyhedrosis virus AcMNPV-beta$_1$-AR (Sf9-beta-$_1$-cells; Fig. 1, **R**), or with wildtype virus (negative control; Fig. 1, **C**) (8) were transferred to nitrocellulose membranes and incubated with serially diluted antibodies for 12 h at 4°C (rabbit anti-beta-$_1$-N/-C/-ECII: 500- to 15,000-fold; mouse IgM: 10- to 1,000-fold; human IgG: 5- to 100-fold). Immunoreactive bands were visualized by appropriate species-specific horseradish peroxidase conjugated secondary antibodies and enhanced chemoluminescence (ECL, Amersham, Buckinghamshire, UK).

For immunoprecipitation, the membrane proteins of Sf9 cells with **(R)** or without **(C)** human beta$_1$-AR were biotinylated with D-biotinoyl-ε-aminocaproic acid (Boehringer), and then precipitated as previously described in detail (8). Briefly, antibodies (two different dilutions: rabbit anti-beta$_1$-N/-C/-ECII, 1:100/250; mouse IgM, 1:10/25; human IgG, 1:5/10) were incubated with the supernatants of solubilized cells for 12 h at 4°C before the addition of γ-bind protein G-Sepharose (Pharmacia, Freiburg, Germany), and for monoclonal IgM antibodies a further secondary goat anti-mouse antibody (Sigma). After incubation of the precipitation reactions for another 2 h at 4°C, protein G-Sepharose was washed six times with PBS, and the adsorbed proteins were eluted with nonreducing 5% SDS-sample buffer. For detection of biotinylated receptors the eluates were transferred to nitrocellulose membranes by Western blotting and probed with horseradish-peroxidase coupled streptavidin (Boehringer). Bound streptavidin was detected by enhanced chemoluminescence (Amersham).

Indirect immunofluorescence microscopy. Immunofluorescence studies on Sf9 cells with **(R)** or without **(C)** human beta$_1$-AR were performed as previously described (7,8). The cells were either transferred to glass slides, fixed with 4% formaldehyde (10 min, 4°C) and permeabilized with 0.1% Triton X-100 (30 s, 4°C), or, when native receptors were analyzed, directly suspended in PBS containing 2 mmol/liter $MgCl_2$ and 2% BSA. For both conditions a single antibody-dilution was utilized (rabbit anti-beta$_1$-N/-C/-ECII 1:200; mouse IgM, 1:50; human IgG, 1:5). After incubation for 1 h at 20°C (fixed and permeabilized cells) or 6 h at 4°C (native intact cells) appropriate species-specific CY3-conjugated secondary antibodies (Dianova, Hamburg, Germany) were added to detect receptor-bound rabbit, mouse, or human antibodies by a Zeiss-Axioplan fluorescence microscope (Carl Zeiss Jena, Oberkochen, Germany).

Radioligand binding. Binding of the radiolabeled antagonist [^{3}H]CGP 12177 (NEN-DuPont, Boston, Massachusetts) to recombinant human beta$_1$-AR expressed in Sf9 cells (1–2 × 10^6 beta$_1$-AR per cell) (7,8) was measured by liquid scintillation counting after incubating 25,000 cells or membranes from 50,000 cells with 3 × 10^{-12} to 5 × 10^{-9} mol/liter [^{3}H]CGP 12177 (30 min, 30°C). Experiments were performed in triplicate on both intact cells and membranes 1) either in the presence or absence of a fixed antibody concentration (mouse, 7 nmol/liter; rabbit, 70 nmol/liter; human IgG, 2 μmol/liter) and various concentrations of [^{3}H]CGP 12177, or 2) at constant 3 nmol/liter [^{3}H]CGP 12177 with various concentrations of the different antibodies (mouse, 7 × 10^{-10} to 7 × 10^{-8} mol/liter; rabbit, 2 × 10^{-9} to 7 × 10^{-7} mol/liter; human, 2 × 10^{-7} to 7 × 10^{-6} mol/liter). Saturation curves were fitted to the data by computer-aided nonlinear regression analysis, assuming binding to a single class of sites with uniform affinity. Nonspecific binding was determined in the presence of 1 μmol/liter unlabeled CGP 12177 and subtracted from the data.

Assays on receptor-mediated cellular signaling (cAMP-assay). The cAMP-assay was carried out on stably transfected Chinese hamster fibroblasts expressing 100–120 fmol/mg human beta$_1$-AR (CHW-beta$_1$ cells). In brief, confluent cells were incubated with the same antibody concentrations as indicated above (radioligand binding) for 1 h at 37°C in the presence of 0.5 mmol/liter isobutylmethylxanthine (IBMX) (7,10,12). After stimulation with 10 μmol/liter (−)isoprenaline (15 min, 37°C) cytoplasmic cAMP was extracted from the cells with boiling water and

Table 2. Titers of Human, Rabbit, and Monoclonal Mouse Anti-beta$_1$-AR Obtained for ELISA, Western Blotting, and Immunoprecipitation

	Antibody					
	Rabbit IgG (Polyclonal, Immunopurified)			Mouse IgM (Monoclonal)	Human IgG (Polyclonal)	
Detection Method	Beta$_1$-N	Beta$_1$-ECII	Beta$_1$-C	Beta$_1$-ECII	Pat. beta$_1$-pos. (n = 8)†	Control (n = 15)†
ELISA						
Antigen reduced*						
Beta$_1$-N	1:32000	—	—	—	—	—
Beta$_1$-ECII	—	1:6400	—	1:100	1:10	—
Beta$_1$-C	1:100	—	1:32000	—	—	—
Beta$_2$-N	—	—	—	—	—	—
Beta$_2$-ECII	—	—	—	—	—	—
Beta$_2$-C	—	—	1:800	—	—	—
Antigen nonreduced						
Beta$_1$-N	1:32000	1:100	—	—	—	—
Beta$_1$-ECII	—	1:32000	—	1:640	1:160	—
Beta$_1$-C	1:100	—	1:32000	1:20	1:20	—
Beta$_2$-N	1:100	—	—	—	—	—
Beta$_2$-ECII	—	1:200	—	1:40	—	—
Beta$_2$-C	—	—	1:1600	—	1:10	—
Western blot	1:10000	1:1000	1:15000	—	—	—
Immunoprecipitation	1:250	1:250	1:250	1:25	1:10	—

*Reduction of the antigen was done by treatment with 1% beta-Me. †One representative example for each of the two subgroups is given. Values given correspond to the maximum dilution at which 1) a statistically significant signal was obtained in ELISA ($p < 0.05$ vs. background, see Methods), or 2) a clear receptor band (Mr 45) was detectable in Western blots or immunoprecipitates (Fig. 1). Negative results at a dilution of 1:100 for rabbit-, 1:10 for mouse-, or 1:5 for human antibodies are indicated by a dash.

Beta$_1$- and beta$_2$-N/-ECII/-C for the N-terminus, second extracellular domain, or C-terminus of the human beta$_1$ or beta$_2$-AR; Pat. beta$_1$-pos. for the subgroup of patients with antibodies able to recognize native beta$_1$-AR (n = 8/30), and Control for IgG-preparations from healthy subjects (n = 15).

measured by ^{125}I-labeled scintillation proximity assay (Biotrak Kit, Amersham). An additional series of cAMP experiments served to analyze the effects of functionally active rabbit (70 nmol/liter) or human anti-beta$_1$-AR (2 μmol/liter) on various states of (agonist-induced) receptor-activity after stimulation of CHW-beta$_1$ cells with increasing amounts of (−)isoprenaline (10^{-12} to 10^{-5} mol/liter). All experiments were repeated three times.

Statistics. The threshold for a significantly increased reactivity in ELISA was based on the statistical distribution (mean ± 2 SD, 95% confidence interval [CI]) of the values obtained for preimmune sera (rabbits, n = 6) or IgG-fractions from healthy control subjects (human, n = 15).

Two-factor analysis of variance (ANOVA) was used to analyze the overall cAMP-response (functional assays). Significance between the antibody subgroups was analyzed by the Scheffé F test (post hoc multiple comparison procedure). A p value <0.05 was considered to be statistically significant. Clinical and hemodynamic parameters of autoantibody-positive and -negative patients were compared by the Student *t* test for unpaired samples. All data are given as mean ± SD.

RESULTS

Immunological characterization of receptor antibodies. Antibodies against the human beta$_1$-AR (anti-beta$_1$-AR) were generated in rabbits and mice, or prepared from the sera of cardiomyopathic patients. Their respective immunoreactivities were determined as previously described (7) 1) by a peptide-based ELISA, 2) by Western blotting, and 3) by immunoprecipitation and immunofluorescence. The results are summarized in Table 2 and Figure 1: The rabbit antibodies directed against the amino (N)- or carboxyterminus (C) of the beta$_1$-AR were equipotent in recognizing their corresponding peptide-antigens in reduced or nonreduced form (Table 2), and clearly stained and/or precipitated intact native human beta$_1$-AR expressed in Sf9 cells (Fig. 1, rabbit, lanes a and b). Antibodies directed against the second extracellular receptor domain were sensitive to reduction of the antigen (Table 2) but were also able to recognize the receptors in Western blots (with a lower signal intensity compared to antibodies against the N- or C-terminus) and to immunoprecipitate the receptors (Fig. 1, rabbit). The monoclonal mouse antibody (of an IgM subtype and lower affinity) directed against the beta$_1$-ECII domain had a similar pattern of immunoreactivity: The antigen treatment with beta-Me led to a decrease in sensitivity for the beta$_1$-ECII peptide (Table 2), and denatured beta$_1$-AR of SDS-treated Sf9-beta$_1$ cells were not stained at all (Fig. 1, mouse, lane a). By contrast, the antibodies could immunoprecipitate the beta$_1$-AR from biotinylated Sf9-cells, giving a streptavidin-positive band on the blots (Fig. 1, mouse, lane b).

From *human* IgG preparations, none of the healthy

control subjects (n = 15) had a significant titer of anti-beta$_1$-AR antibodies, whereas 47% of the cardiomyopathic patients (n = 14/30) had autoantibodies able to bind to synthetic peptide-antigens corresponding to selected domains of the human beta$_1$- or beta$_2$-AR (not shown). However, only a subgroup of these patients (n = 8/14) had functionally relevant anti-beta$_1$-AR able to precipitate native human beta$_1$-AR from cell membranes (Fig. 1, human, line b). None of them recognized SDS-denatured beta$_1$-AR on Western blots (Fig. 1, human, lane a), which is in agreement with our previous results (7). All of these antibodies showed increased reactivity with the beta$_1$-ECII peptide-antigen, but only in its nonreduced form (Table 2).

Taken together, these results indicate that anti-beta$_1$-ECII preferentially or exclusively recognize a conformational epitope within the ECII-domain of the beta$_1$-AR, which can be denatured by reducing agents or by SDS treatment. This notion was further supported by immunofluorescence experiments: The Sf9-beta$_1$ cells were either fixed and permeabilized (Fig. 1, lane c), or untreated cells were directly incubated with the different antibodies (Fig. 1, lane d). None of the rabbit, mouse or human anti-beta$_1$-ECII recognized beta$_1$-AR on fixed Sf9 cells, whereas all of them immunostained the receptor, when it was natively presented on intact Sf9-beta$_1$ cells. In contrast, rabbit anti-beta$_1$-N stained both fixed and unfixed Sf9-beta$_1$ cells with the same intensity, and rabbit anti-beta$_1$-C stained only fixed, permeabilized Sf9-beta$_1$ cells (Fig. 1, rabbit, lanes c and d). The IgG preparations from healthy subjects gave negative results throughout in both Western blots and immunoprecipitation and/or fluorescence experiments (not shown).

Interference of receptor antibodies with ligand binding. The results from radioligand-binding studies brought further evidence for the recognition of a native receptor conformation by anti-beta$_1$-ECII. Only these antibodies were able to decrease binding of [^{3}H] CGP 12177 to recombinant human beta$_1$-AR in a concentration-dependent manner (Fig. 2a): The antibodies raised in rabbits or mice were more potent inhibitors compared to anti-beta$_1$-ECII from cardiomyopathic patients and decreased [^{3}H] CGP 12177 binding by 55 ± 3% (mean ± SD; rabbit IgG, 700 nmol/liter) or 48 ± 5% (mouse IgM, 70 nmol/liter) compared to only 24 ± 11% by human receptor autoantibodies (patient IgG, 7 μmol/liter). Similar results were obtained for intact Sf9-beta$_1$ cells and membrane preparations from these cells (not shown).

The [^{3}H] CGP 12177 saturation curves in the presence of a fixed concentration of anti-beta$_1$-ECII antibodies (mouse, 7 nmol/liter; rabbit, 70 nmol/liter; human, 2 μmol/liter) revealed that all of them decreased total ligand binding capacity (B_{max}) by about the same extent (from 8.2 ± 0.1 [mean ± SD; control] to 6.3 ± 0.3 [rabbit], 6.2 ± 0.2 [mouse], or 5.9 ± 0.4 pmol/mg [human], respectively, $p < 0.001$; Fig. 2b). Interestingly, human and rabbit anti-beta$_1$-ECII also decreased [^{3}H] CGP 12177 affinity

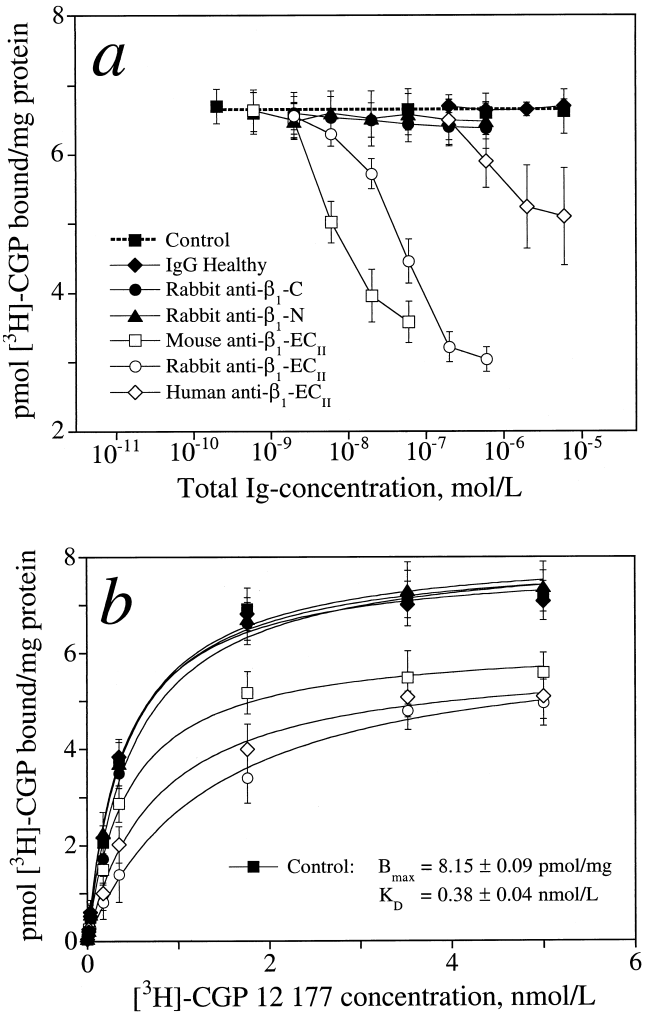

Figure 2. Decrease in ligand-binding to human beta$_1$-AR expressed in Sf9 cells **(a)** in the presence of constant 3 nmol/liter [^{3}H]CGP 12177 and the indicated concentrations of anti-beta$_1$-AR, or **(b)** in the presence of a fixed concentration of the different anti-beta$_1$-AR (mouse, 7 nmol/liter; rabbit, 70 nmol/liter; human: 2 μmol/liter) and various concentrations of [^{3}H]CGP 12177 (3×10^{-11} to 5×10^{-9} mol/liter). Error bars indicate the mean ± SD of three experiments. B_{max} = total ligand binding capacity; K_D = receptor-ligand dissociation constant.

(K_D) by about 2- or 4-fold (from 0.38 ± 0.04 [mean ± SD; control] to 0.74 ± 0.1 [human] or 1.34 ± 0.02 nmol/liter [rabbit], respectively) indicating some degree of allosteric competition between the radioligand and these antibodies. By contrast, rabbit anti-beta$_1$-N/-C and IgG-preparations from autoantibody-negative patients and/or healthy control subjects did not significantly affect [^{3}H] CGP 12177 binding to the beta$_1$-AR (Fig. 2b).

Effects of receptor antibodies on receptor-mediated signaling. Antibody effects on transmembrane signaling were analyzed on stably transfected CHW-beta$_1$ cells (7,12). Only anti-beta$_1$-ECII were able to modulate intracellular cAMP levels (Fig. 3); however, their effects on receptor-mediated signaling differed considerably (Table 3): The rabbit and mouse anti-beta$_1$-ECII significantly decreased both basal and isoprenaline-stimulated cAMP accumulation

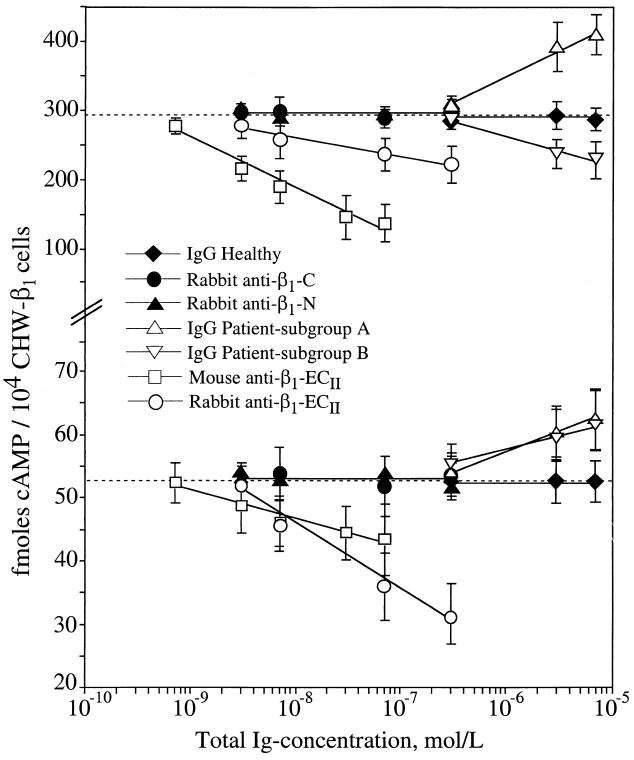

Figure 3. Increase in basal or (−)isoprenaline (10 μmol/liter)-stimulated cAMP-levels upon incubation of CHW-beta$_1$ cells with the indicated concentrations of rabbit, mouse or human anti-beta$_1$-ECII **(open symbols)**, or rabbit anti-beta$_1$-C/-N **(filled circles/triangles)**. IgG-preparations from healthy subjects served as a control **(filled diamonds)**. Error bars indicate the mean ± SD of three experiments.

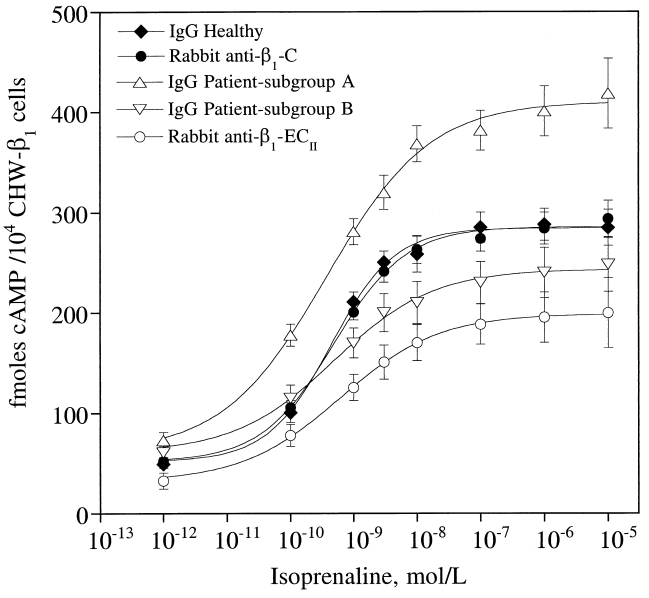

Figure 4. Increases in (−)isoprenaline-stimulated cAMP-levels (10^{-12} to 10^{-5} mol/liter) upon incubation of CHW-beta$_1$ cells with a fixed concentration of rabbit (70 nmol/liter) or human anti-beta$_1$-ECII (2 μmol/liter; patient-subgroups A and B). **Filled circles** or **diamonds** correspond to the values obtained for rabbit anti-beta$_1$-C or IgG-preparations from healthy subjects, respectively. Error bars indicate the mean ± SD of three experiments. Significance between control values **(filled symbols)** and those obtained in the presence of anti-beta$_1$-ECII **(open symbols)** was analyzed by the Scheffé F test. All the values obtained for patient anti-beta$_1$-ECII (subgroups A/B) differed significantly ($p < 0.05$) from control values in the presence of low (10^{-12} to 10^{-10} mol/liter) or high (10^{-9} to 10^{-5} mol/liter) isoprenaline concentrations, except those from patient subgroup B at 10^{-10} mol/liter isoprenaline ($p = 0.15$, not significant).

in a concentration-dependent manner, and this inhibition was particularly evident at higher agonist concentrations (Fig. 4, rabbit anti-beta$_1$-ECII). Because these antibodies inhibited not only the agonist-stimulated but also the basal cAMP production, their functional properties might be best characterized as inverse agonists. By contrast, the majority of the anti-beta$_1$-ECII autoantibodies from cardiomyopathic patients (n = 6/8, patient-subgroup A) increased basal ($p < 0.02$) and even further increased agonist-stimulated cAMP production (on top of the stimulation obtained with 10 μmol/liter (−)isoprenaline, $p < 0.002$; Fig. 3 and Table 3). Again, this effect appeared to be more pronounced at higher agonist concentrations (Fig. 4, patient-subgroup A).

Interestingly, two of the autoantibody-positive patients (n = 2/8, patient-subgroup B) had anti-beta$_1$-ECII, which affected beta$_1$-AR activity in a manner that could be placed between these two opposite fashions of modulating receptor signaling. These antibodies *in*creased basal but *de*creased isoprenaline-stimulated cAMP accumulation dependent on the degree of receptor activation (Fig. 4): In the presence of very low amounts of agonist intracellular cAMP production was still increased (10^{-12} mol/liter (−)isoprenaline; $p < 0.05$), whereas it was inhibited at higher agonist-

Table 3. Basal and (−)Isoprenaline-Stimulated cAMP-levels of CHW-beta$_1$ Cells in the Presence of Rabbit (70 nmol/liter), Mouse (7 nmol/liter), or Human Anti-beta$_1$-ECII (2 μmol/liter)†

		Anti-beta$_1$-ECII Antibodies			
				Human	
Assay Condition	Control IgG	Rabbit	Mouse	Subgroup A	Subgroup B
Basal	52 ± 4	32 ± 5*	43 ± 6*	63 ± 4*	61 ± 6*
+ 5 μmol/liter bisoprolol	51 ± 5			54 ± 6	53 ± 7
10 μmol/liter (−)isoprenaline	294 ± 18	223 ± 31**	138 ± 26**	418 ± 35**	228 ± 27*
+5 μmol/liter bisoprolol	56 ± 5			57 ± 9	58 ± 7

†Values are mean ± SD, given in [fmoles cAMP/10^4 CHW-beta$_1$ cells]. Significance between the antibody subgroups was analyzed by the Scheffé F test. Significant differences (compared to control IgG) are denoted by *$p < 0.05$; **$p < 0.005$.

concentrations (10^{-9} to 10^{-5} mol/liter (−)isoprenaline; $p < 0.05$ to $p < 0.02$). Thus, their functional properties might be compared with those of a partial agonist. All functional effects of agonist- and/or partial agonist-like human anti-beta$_1$-ECII were abolished in the presence of 5 μmol/liter bisoprolol, indicating that the antibodies acted, indeed, via the beta$_1$-AR (Table 3).

Although not a main issue of this study, an analysis of the clinical and hemodynamic parameters of our patient-collectives revealed that left ventricular function was significantly more reduced in anti-beta$_1$-ECII-positive (n = 8) than in antibody-negative (n = 22) DCM-patients (Table 1). However, because of the restricted number of antibody-positive patients in the present study we were not able to determine specific clinical (i.e., age, duration of disease, medication) or hemodynamic (i.e., heart rate, left ventricular ejection fraction, cardiac index) features related to the prevalence of agonist-like (subgroup A) or partial agonist-like (subgroup B) anti-beta$_1$-ECII autoantibodies.

DISCUSSION

Anti-beta-receptor antibodies and receptor function. Antibodies against certain G protein-coupled receptors can affect receptor signaling, and such effects have been postulated to play a pathophysiological role in several human diseases (1–7,18,19). The present study shows that in the case of the beta$_1$-AR, only those antibodies that were directed against the ECII-loop domain (anti-beta$_1$-ECII) recognized a native receptor conformation in a variety of assays (ELISA, immunoprecipitation/-fluorescence), and that the same antibodies also affected receptor function. In contrast, antibodies directed against the N- or C-terminus were not sensitive to denaturation of the receptor and had no effects on receptor function.

The ligand binding pocket of beta-AR is thought to be formed by the transmembrane helices. In the case of the beta$_2$-AR, amino acids in helices III, V, and VI have been assigned an anchoring function for agonists (20,21). These data suggest that extracellular loops do not directly participate in ligand binding; however, reduction or mutations of cysteine residues in the extracellular loops of the beta$_2$-AR, most notably the ECII-domain, result in a reduction of agonist and antagonist affinities (22). Thus, correct folding of this loop may be essential for correct formation of the ligand binding pocket and might explain, why anti-beta$_1$-ECII can affect ligand binding.

In addition, such antibodies can also affect receptor function, and these functional effects are of a very different nature for different antibodies, suggesting that they act as allosteric regulators of receptor activity: Antibodies binding to the ECII-loop might reduce or stabilize conformational changes of the receptors, which are similar to those induced by agonist or partial agonist ligands (1,9,18,20–23). All of our anti-beta$_1$-ECII interact with the rather small stretch of amino acids that form this loop but have different effects on receptor function. One possible explanation for this divergence is that they recognize the receptor preferentially (rabbit) or exclusively (mouse, human) in a native conformation; it is possible that among the native conformations some antibodies recognize the active and others the inactive form (1,18). With exception of the mouse antibody, because of the anti-beta$_1$-ECII described here are polyclonal, they may in fact recognize different conformational states within the targeted receptor domain. Finally, our data suggest that both the subtype (i.e., IgG or IgM) and the source of an immunoglobulin (i.e., the species it stems from) may influence its respective functional properties as well.

Allosteric effects of antibodies against the second extracellular receptor domain. The functional effects of anti-beta$_1$-ECII may be discussed according to a model (23) that assumes two states for receptors, inactive **(R)** and active **(R*)**. Agonists **(A)** can bind to both states, but induce and/or stabilize the active state, forming preferentially **AR***. Antibodies (= immunoglobulins, I) can bind to the active **(IR*)** or inactive **(IR)** states, and can also do so in the presence of agonist. Our mouse and rabbit anti-beta$_1$-ECII reduced the amount of active receptors, probably by stabilizing an inactive state **IR**. In the presence of agonist, they have a similar inhibitory effect, either because they reduce agonist binding (again forming **IR**), or because they return the receptors to an inactive state even with agonist bound **(AIR)**. These two antibodies behave as inverse agonistic allosteric regulators, because they inhibit basal as well as stimulated receptor activities. In contrast, all of the functionally active human anti-beta$_1$-ECII increased basal receptor activity, presumably by forming an active state **(IR*)**. Again, they may do so by preferentially binding to the active state **R*** (as suggested for antibodies against the beta$_2$-AR [18]), or by inducing an active receptor conformation **(IR*)**. In the presence of agonist, most human anti-beta$_1$-ECII autoantibodies caused a further increase in activity (patient-subgroup A), whereas only a minor fraction decreased agonist-induced beta$_1$-AR activity (patient-subgroup B). The allosteric effects caused by the latter antibodies appear comparable to those of a partial agonist. Antibodies from patient-subgroup A may either induce a state **AIR*,** which is more active than the agonist-activated state **AR*,** or alternatively, cause more receptors to switch into the active state than even high agonist concentrations do.

Conclusions and perspectives. In summary, antibodies recognizing a same small domain of the beta$_1$-AR can have very divergent functional effects. A subgroup of patients suffering from certain cardiac disorders has been shown to have circulating autoantibodies to the human beta$_1$-AR (5–7,12), and like those of the patient-subgroup A reported here, some of these antibodies appear to have agonist-like properties and/or act as receptor-sensitizing agents. In the present study, we focused on patients with DCM; however, it should be remembered that functionally active anti-beta$_1$-ECIIs have also been found in ischemic cardiomyopathy (ICM), although with less frequency (7). Because antibody-

induced adrenergic (over-)stimulation might contribute to worsening cardiac dysfunction in both cases (7) sensitizing anti-beta$_1$-ECII may represent a negative prognostic factor in DCM and/or ICM. In agreement with this hypothesis, the antibody-positive DCM-patients here analyzed had a significantly poorer left ventricular function compared to those without such antibodies (Table 1); however, because of the restricted number of antibody-positive patients, we were not able to identify clinical features characteristic for type A (agonist-like) or type B (partial agonist-like) anti-beta$_1$-AR autoantibodies. In the following it will be interesting to study, whether these antibody subtypes are related to clinical (and/or hemodynamic) parameters, and whether they are of predictive value.

Reprint requests and correspondence: Dr. Roland Jahns, Medizinische Poliklinik, University of Wuerzburg, Klinikstrasse 6-8, D-97070 Wuerzburg, Germany. E-mail: Jahns@wpxx02.toxi.uni-wuerzburg.de.

REFERENCES

1. Fraser CM, Venter JC. Anti-receptor antibodies in human disease. J Allergy Clin Immunol 1984;74:661–73.
2. Endo K, Kasagi K, Konishi J, et al. Detection and properties of TSH-binding inhibitor-immunoglobulins in patients with Graves' disease and Hashimoto's thyroiditis. J Endocrinol Metab 1978;46:734–9.
3. Tzartos SJ, Seybold ME, Lindstrom JM. Specificities of antibodies to acetylcholine receptors in sera from myasthenia gravis patients measured by monoclonal antibodies. Proc Natl Acad Sci U S A 1982;79:178–92.
4. Harrison L, Van Obberghen E, Grunfield C, King G, Khan C. Modulation of the insulin receptor by insulin receptor autoantibodies. In: Cohen E, Kohler H, editors. Membrane Receptors and the Immune Responses. New York: Alan R. Liss, 1980:109–26.
5. Wallukat G, Morwinski M, Kowal K, Förster A, Boewer V, Wollenberger A. Autoantibodies against the β-adrenergic receptor in human myocarditis and dilated cardiomyopathy: β-adrenergic agonism without desensitization. Eur Heart J 1991;12:178–81.
6. Magnusson Y, Wallukat G, Waagstein F, Hjalmarson Å, Hoebeke J. Autoimmunity in idiopathic dilated cardiomyopathy. Characterization of antibodies against the β_1-adrenoceptor with positive chronotropic effect. Circulation 1994;89:2760–7.
7. Jahns R, Boivin V, Siegmund C, Inselmann G, Lohse MJ, Boege F. Autoantibodies activating human β_1-adrenergic receptors are associated with reduced cardiac function in chronic heart failure. Circulation 1999;99:649–54.
8. Jahns R, Siegmund C, Jahns V, et al. Probing human β_1- and β_2-adrenoceptors with domain-specific fusion protein antibodies. Eur J Pharmacol 1996;316:111–21.
9. Lohse MJ, Strasser RH, Helmreich EJM. The β-adrenoceptors. In: Hucho F, editor. Neurotransmitter Receptors. Amsterdam: Elsevier Science Publishers, 1993:137–80.
10. Klotz K-N, Hessling J, Hegler J, et al. Comparative pharmacology of human adenosine receptor subtypes—characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedebergs Arch Pharmacol 1998;357:1–9.
11. Frielle T, Collins S, Daniel KW, Caron MG, Lefkowitz RJ, Kobilka BK. Cloning of the cDNA for the human β_1-adrenergic receptor. Proc Natl Acad Sci U S A 1987;84:7920–4.
12. Jahns R, Boivin V, Siegmund C, Boege F, Lohse MJ, Inselmann G. Activating β_1-adrenoceptor antibodies are not associated with cardiomyopathies secondary to valvular or hypertensive heart disease. J Am Coll Cardiol 1999;34:1545–51.
13. Wallukat G, Reinke P, Dörffel WV, et al. Removal of autoantibodies in dilated cardiomyopathy by immunoadsorption. Cardiology 1996;54:191–5.
14. Kobilka BK, Dixon RA, Frielle T, et al. cDNA for the human β_2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. Proc Natl Acad Sci U S A 1987;84:46–50.
15. Kearney JF, Radbruch A, Liesegang B, Rajewski K. A mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting cell lines. J Immunol 1979;123:1548–58.
16. Galfre G, Howe HC, Milstein C, Butcher GW, Howard JC. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 1977;266:550–2.
17. Coller HA, Coller BS. Poisson statistical analysis of repetive subcloning by the limiting dilution technique as a way of assessing hybridoma monoclonality. In: Langone JJ, Van Vunakis H, editors. Methods in Enzymology. New York: Academic Press, 1983;412–7.
18. Mijares A, Lebesgue D, Argibay J, Hoebeke J. Anti-peptide antibodies sensitive to the "active" state of the β_2-adrenergic receptor. FEBS Lett 1996;399:188–91.
19. Lohse MJ. G-proteins and their regulators. Naunyn Schmiedebergs Arch Pharmacol 1999;360:3–4.
20. Strader CD, Sigal IS, Dixon RA. Structural basis of β-adrenergic receptor function. FASEB J 1989;3:1825–32.
21. Wieland K, Zuurmond HM, Andexinger S, Ijzerman AP, Lohse MJ. Stereospecificity of agonist binding to β_2-adrenergic receptors involves Asn-293. Proc Natl Acad Sci U S A 1996;93:9276–81.
22. Dohlman HG, Caron MG, De Blasi A, Frielle T, Lefkowitz RJ. Role of extracellular disulfide-bonded cysteines in the ligand binding function of the β_2-adrenergic receptor. Biochemistry 1990;29:2335–42.
23. Milligan G, Bond A. Inverse agonism and the regulation of receptor number. Trends Pharmacol Sci 1997;18:468–74.