Activating Beta-1-Adrenoceptor Antibodies Are Not Associated With Cardiomyopathies Secondary to Valvular or Hypertensive Heart Disease

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OBJECTIVES
We investigated whether autoantibodies against the human beta-adrenergic receptor (beta-AR) might be involved in cardiomyopathies secondary to valvular heart disease (VHD) or hypertensive heart disease (HHD).

BACKGROUND
Autoimmunity to beta-AR has been proposed as a pathogenic principle in human cardiomyopathy. Recently, by the use of intact recombinant human beta-AR, we were able to confirm the existence of functionally active anti-beta-1-AR autoantibodies in patients with dilated cardiomyopathy (26% prevalence) or ischemic cardiomyopathy (10% prevalence); however, their prevalence in other (secondary) cardiomyopathies remained to be determined.

METHODS
Immunoglobulin G (IgG) was prepared from the sera of 28 VHD and 19 HHD patients and first screened by a peptide-based enzyme-linked immunosorbent assay (antigens: aminoterminal, second extracellular loop [ECII] and carboxyterminus of human beta-1- and beta-2-AR). IgG from 108 gender- and age-matched healthy subjects served to define the threshold for positive immunoreactions. Positive sera were further screened for their ability to recognize and activate native human beta-AR situated in a cell membrane.

RESULTS
Twenty-five percent (VHD) or 11% (HHD) of the patients and 4% of the healthy controls had IgG antibodies randomly directed against all the three domains tested and both beta-AR subtypes. Only one patient with aortic valve and concomitant coronary heart disease and one healthy subject had functionally active anti-b1-AR (targeting beta-1-ECII). Moreover, one HHD patient with concomitant collagenosis had IgG that was cross-reacting with recombinant beta-AR in immunological assays but was unable to affect receptor function.

CONCLUSIONS
Autoimmune reactions against the human beta-AR do not appear to be associated with cardiomyopathies secondary to VHD or HHD. (J Am Coll Cardiol 1999;34:1545–51) © 1999 by the American College of Cardiology

Since the first reports on the detection of stimulating anti-beta-adrenergic receptor (anti-beta-AR) autoantibodies in patients with Chagas cardiomyopathy (1), a growing body of evidence suggests that autoimmunity to cardiac beta-ARs might be associated with several other human cardiomyopathies as well (2,3). Recently, we established a screening strategy for detecting functionally relevant anti-beta-AR in patients suffering from dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) by the use of recombinant intact human beta-AR expressed in various cell types (4). Previously generated subtype- and domain-specific anti-beta-AR rabbit antibodies enabled us to define positive immunoreactions (5). Confirming the results from earlier studies (2,3), in both DCM and ICM we detected a subset of patients with circulating immunoglobulin G (IgG) antibodies able to recognize native human beta-1-AR in a cell membrane and activate and/or sensitize these receptors through a direct (allosteric) receptor-antibody interaction. Their prevalence was 26% in DCM and 10% in ICM, respectively, and was associated with a significant reduction in cardiac function (4).

However, in other kinds of heart disease also accompanied by reduced left ventricular function (and finally severe heart failure), the prevalence and possible implication of such activating anti-beta-1-AR remained to be evaluated. Consequently, in the present study, we used the newly established tools to screen the sera of 47 patients suffering from cardiomyopathies secondary to valvular (valvular heart disease [VHD], n = 28) or hypertensive heart disease ([HHD], n = 19) for functionally active autoantibodies targeting the human beta-AR.
METHODS

Patients and sample acquisition. Forty-seven patients were recruited in the course of routine heart catheterization and coronary angiography: 28 patients suffered from chronic VHD (VHD subgroup; Table 1); 19 patients had a long-lasting clinical history of hypertension (22 ± 9 years) and suffered from advanced states of HHD (HHD subgroup). All patients had dyspnea corresponding to New York Heart Association functional classes II to IV, a left ventricular diastolic volume >110 ml/m² and an ejection fraction <55% (ventriculography). For HHD patients, significant coronary heart disease could be excluded by angiography, whereas three patients from the VHD subgroup had concomitant beginning (one-vessel disease, less than 50% stenosis), and one VHD patient had severe coronary artery disease but no prior infarction in clinical history. Because this patient had significant aortic valve stenosis (accompanied with severe aortic valve regurgitation) but no pathological Q-waves in the electrocardiogram, he was assigned to the VHD subgroup. At the time of sample acquisition, all patients were stable under therapy with diuretics, angiotensin converting enzyme inhibitors, digitalis or nitrates; five patients also received amiodipine. None was treated with beta-receptor blockers or beta-receptor agonists. One hundred eight healthy blood donors matched for gender (female/male = 38/70) and age (48 ± 18 years) served as controls (collective identical to our previous study [4]). Table 2 summarizes the basic clinical and hemodynamic data of the patient subgroups analyzed.

IgG was prepared from the sera by caprylic acid precipitation; the concentration was determined by immuno nephelometry (BN-II; Dade-Behring, Marburg, Germany), and for the assays, all samples were normalized to 1,000 µg IgG/ml.

Immunoassays with synthetic antigens. For enzyme-linked immunosorbent assay (ELISA), synthetic peptides corresponding to the second extracellular loop of the human beta-1 or beta-2-AR were synthesized commercially (ami-
no acids 195 to 225 or 169 to 200, according to the amino acid sequences of the human beta-1- [6] or beta-2-AR [7], respectively). Amino- and carboxy-terminal fusion proteins of both receptor subtypes were produced in *Escherichia coli* as described previously (5), and fragments corresponding to the respective receptor-domains were isolated by high-performance liquid chromatography (HPLC) after thrombin digestion of the fusion products (to cleave off the bacterial fusion partner). Peptide (5 ng/well) or protein were coated onto 96-well microtiter plates (Maxisorb; Nunc, Kastrup, Denmark), incubated with two different IgG concentrations (25 and 12.5 μg/ml, each in triplicate) for 12 h at 4°C and washed six times before the addition of biotinylated secondary rabbit anti-human IgG antibodies (1:5,000), streptavidin-peroxidase and o-phenylenediamine (Sigma, Deisenhofen, Germany). Optical densities were read at 490 nm.

Dot-blots were obtained using the same antigens as above, spotted onto activated nitrocellulose membranes (8) (Hybond C; Amersham, Buckinghamshire, UK). In addition to the selected receptor fragments, in this assay the IgG fractions were also checked against spots of bovine serum albumin (BSA) and a nonreceptor control peptide. The membranes were probed with 50 μg/ml of the human IgG preparations for 2 h at 37°C, washed eight times and after incubation with horseradish peroxidase-conjugated goat anti-human IgG secondary antibodies, immunoreactive dots were visualized by enhanced chemiluminescence (ECL; Amersham).

**Immunoaassays with intact recombinant receptors.** Whole cell lysates or intact unfixed Sf9 insect cells infected with recombined baculovirus AcMNPV-beta-1-AR or -beta-2-AR transiently expressing 1 to 2 × 10^6 human beta-1- or beta-2-AR (4,9) were utilized for native Western blotting and immunofluorescence studies. Wild-type virus-infected Sf9 cells served as negative controls. Presence of the receptor antigens was checked with previously characterized subtype-specific rabbit anti-beta-AR (positive control) (5). Sf9 cell lysates were subjected to native Western blotting as previously described (4,10) and incubated with 50 μg/ml of the patient IgG for 12 h at 4°C. Immunoreactive bands were visualized by enhanced chemiluminescence (see above).

Immunofluorescence experiments were performed on intact unfixed Sf9 cells, suspended in phosphate-buffered saline (PBS) containing 2 mmol/liter MgCl_2 and 2% BSA. The cells were incubated with human IgG fractions (167 and 83 μg/ml) for 6 h at 4°C and then washed three times before the addition of CY3-labeled anti-human IgG secondary antibodies (Dianova, Hamburg, Germany) to detect receptor-bound patient IgGs. Fluorescence of the cells was inspected by a fluorescence microscope (400-fold magnification; Zeiss-Axioplan, Jena, Germany) and photographed with identical exposure times.

**Assays on receptor-mediated cAMP accumulation.** The cAMP assay was carried out on stably transfected Chinesehamster fibroblasts expressing 100 to 120 fmol/mg human beta-1-AR (CHW-beta-1 cells) (4). Cellular cAMP was determined by 12^5^I-labeled cAMP scintillation proximity assay (Biotrak Kit; Amersham) in three independent experiments. In brief, confluent cells were preincubated with 40 μg IgG/ml from patients and healthy controls for 1 h at 37°C in the presence of 0.5 mmol/liter isobutylmethylxanthine. Subsequently, the cells were either stimulated with 10 μmol/liter (-)isoproterenol or not, and incubation continued for 15 min at 37°C. After washing the cells two times with medium, cytoplasmic cAMP was extracted with boiling water, and the supernatants were directly used for the scintillation assay.

**Statistical analysis.** The threshold for a significantly increased reactivity in ELISA was based on the statistical distribution of n = 108 gender- and age-matched healthy control subjects (mean ± 2 SD). Statistical analysis of the cAMP assay was done by two-factorial ANOVA, using the experimental condition and the disease entity as factors (F = 2.032 [basal vs. stimulated] and F = 13.8 [intergroup differences, respectively; p < 0.001). Subgroup analysis was done by Scheffé’s F-test (post hoc multiple comparison procedure). The observed power (beta-1) of the method was superior to 99.9% (functional assay). Recently, by using the same approach, we were able to detect n = 17/65 DCM and n = 4/39 ICM patients who had: 1) IgG antibodies activating cellular cAMP production and 2) significantly reduced cardiac function (Cardiac index 2.21 ± 0.1 vs. 2.81 ± 0.1 liters/min/m²; p < 0.001 [4]).

**RESULTS**

**Screening with synthetic beta-adrenergic receptor peptides.** Sera of patients suffering from VHD or HHD and healthy controls were initially screened for IgG antibodies capable of binding to synthetic peptides corresponding to the aminoterminus, second extracellular loop, or carboxyterminus of human beta-1- and beta-2-AR, as previously described (4). An increased reactivity in ELISA was assumed for signals above the upper limit of the respective 95% confidence intervals (mean ± 2 SD) of the healthy subjects. By this criterion, 36% of the patient IgG (39% of the VHD and 32% of the HHD subgroup) revealed increased reactivity with at least one of the selected receptor domains (Fig. 1, left). Fifty-nine percent of the positive sera showed either multiple reactivity with different receptor domains (Fig. 1, numbers in parentheses) or cross-reactions between the beta-1- and beta-2-receptor subtype (Fig. 1, hatched bars). This high prevalence of multiple reactivity indicated either a polyclonal immune response against the whole receptor molecule or high levels of nonspecific binding.

In order to select IgG antibodies specifically binding to the above synthetic receptor fragments, all IgG fractions with increased reactivity in ELISA were further checked for nonspecific binding to BSA and to a nonreceptor control
peptide by dot-blotting (not shown). The number of patients still remaining antibody-positive indicated that more than half of the multiple reactions with several receptor domains were obviously due to nonspecific binding: 25% (n = 7/28) and 11% (n = 2/19) of the VHD and HHD patients, respectively, and less than 4% of the healthy subjects (n = 4/108), had circulating antibodies specific for synthetic beta-AR domains. These immunoreactive IgGs were directed with similar frequency against all three domains tested and both beta-AR subtypes.

**Immunofluorescence-studies on native human beta-adrenergic receptors.** Immunoreactive IgG specifically binding to synthetic receptor fragments was further checked for their capability of recognizing intact native human beta-AR. Only two of the patient IgG preparations (one from either subgroup) stained the membranes of Sf9 cells bearing beta-AR in their native conformation. These IgG antibodies were the ones directed against the second extracellular loop of the beta-1-AR subtype (anti-b1-ECII). However, none of the antibodies was able to recognize the receptors on renatured Western blots of Sf9 cell lysates, suggesting that anti-b1-ECII preferentially recognize a specific native receptor conformation that is sensitive to denaturation and cannot be completely restored after Western blotting. Figure 2 shows the typical membrane staining pattern of Sf9 cells transiently expressing human beta-1- (top) or beta-2-AR (middle) upon incubation with subtype-specific anti-beta-AR rabbit antibodies (Fig. 2, rabbit anti-b1/b2), with IgG from an antibody-negative healthy subject (Healthy), or with IgG from a patient with chronic valvular and concomitant ischemic heart disease recognizing only the beta-1-AR (VHD), or a patient with hypertensive heart disease and concomitant collagenosis recognizing both beta-AR bearing Sf9 cells and wild-type virus-infected control cells (nonspecific staining, HHD).

**Figure 1.** Screening of patients with VHD or HHD, or healthy controls (Healthy) for anti-beta-AR autoantibodies. (Left) Increased reactivity in ELISA with synthetic peptides corresponding to the aminoterminal (N), second extracellular (ECII), or carboxyterminal (C) domains of human beta-1- (open bars), or beta-2- (closed bars), or both receptor subtypes (hatched bars). (Right) Sera specifically recognizing receptor peptides selected for binding (IFM = indirect fluorescence microscopy, see Fig. 2) and functional interaction (increase in cAMP, see Fig. 3) with intact human beta-AR. Bars represent the number of positive results for each epitope; numbers in parentheses (on top of the bars) indicate cross-reactions with another receptor domain. The total number of autoantibody-positive individuals is given underneath each diagram (brackets).
combined aortic valve disease. However, left heart catheterization and coronary angiography revealed that this patient at the same time suffered from significant stenosis of the proximal left descending anterior artery and of the proximal portion of the left circumflex artery. The ventriculogram showed diffuse reduction in wall motion, suggesting concomitant ischemic cardiomyopathy (left ventricular ejection fraction 15%; cardiac index 1.7 liters/min/m²).

None of the IgG preparations from healthy subjects who were not reacting with synthetic receptor peptides in ELISA (Fig. 2, Healthy), or from patients and controls recognizing only the amino- and/or carboxyterminal receptor domains, stained native beta-AR expressed in Sf9 cells. However, 1 out of 108 healthy control subjects could be identified who had antibodies that recognized both the beta-1-ECII peptide and the native intact beta-1-AR expressed in Sf9 cells (Fig. 1, Healthy, right). At the time of sample acquisition, this individual had no cardiac abnormalities (as assessed by echocardiography and electrocardiogram) and no pathological findings in the routine blood chemistry, including a screening for antinuclear antibodies.

**Antibody effects on receptor-mediated cellular signaling.** All immunoreactive IgG fractions were also tested for possible functional effects by the use of stably transfected CHW-b1 cells. Only the IgG antibodies from the fluorescence-positive VHD patient and those from the only positive healthy control subject (able to recognize and to bind to native human beta-1-AR) affected receptor-mediated signaling (Fig. 3). In the presence of these antibodies, basal cAMP levels were significantly increased by 1.48- or 1.19-fold (Healthy positive), and isoproterenol-stimulated cAMP production was also increased by 1.39 ± 0.08- or 1.09 ± 0.04-fold, respectively (Fig. 3b). These data confirm our previous findings, that functionally relevant human anti-beta-1-AR may enhance receptor-mediated cAMP accumulation (4). By contrast, the IgG preparations from all the other healthy controls (n = 15) and patients (n = 17) analyzed in the frame of the present study, including those of the HHD patient nonspecifically staining the membranes of beta-AR expressing Sf9 cells, had no effect on basal or agonist-stimulated cAMP production in repeated experiments (Fig. 3, Healthy negatives and HHD patient).

**DISCUSSION**

The second extracellular loop of the beta-1-AR as autoantigen. Since the first reports on circulating anti-beta-AR autoantibodies in cardiomyopathy, several groups have investigated the possible role and functional implication of receptor-directed autoimmune reactions in the development and/or course of human heart disease (2,3). However, depending on the respective screening strategies and immunological detection methods employed, the reports on the prevalence and/or functional impact of such receptor antibodies differed to some extent (2,3,11). Recently, by using the molecular tools developed during the past few years (5–7,9), we were able to confirm the existence of functionally active human anti-beta-1-AR in a subgroup of patients suffering from DCM or ICM (4). Similar to those from the antibody-positive (VHD) patient in the present study, all of these antibodies were directed against the fairly small second extracellular loop domain of the human beta-1-AR (anti-beta-1-ECII), which is in agreement with previous findings from a Swedish group (2). Although this loop is probably not directly involved in receptor-ligand binding (12), because of its disulfide-bonded cysteines, it might be important for the correct folding of the receptor molecule: 1) for ligand accessibility (13) or 2) for transition into an active receptor state (as demonstrated for the beta-2-AR [14]). In addition, this loop has been shown to contain epitopes capable of stimulating immunoreactive B- and T-cells in vivo (15), which could explain its possible role as autoantigen.

The positive staining of beta-1-AR in native cells and the negative results of Western blots suggest that anti-b1-ECII antibodies preferentially bind to the receptor in its native conformation. Thereby, they appear to be able to increase both basal and isoproterenol-stimulated intracellular second messenger (cAMP) levels. Such functionally active anti-beta-1-AR antibodies might act by promoting an active receptor conformation and, thus, sensitize the beta-adrenergic system for catecholamines, which in a failing heart could further potentiate the vicious circle of adrenergic overdrive (16). In agreement with this hypothesis, the only activating antibody-positive (VHD) patient identified...
here suffered from severe cardiac dysfunction (cardiac index 1.7 liters/min/m²), and in our previous study on patients with DCM and ICM, the prevalence of activating anti-beta-1-AR was also strongly associated with a more reduced left ventricular function (4). Thus, it can be imagined that autoantibody-mediated activation of beta-1-AR might have adverse effects on the clinical course of a certain subset of human cardiomyopathies, similarly to a number of other receptor-targeted autoimmune diseases in which autoantibodies have been shown to influence the progression of disease (17).

Receptor-antibodies in valvular and HHD. The results from our study indicate that activating anti-beta-AR autoantibodies are unlikely to play a major role in cardiomyopathies secondary to VHD or HHD, which would be in agreement with recent findings from other groups suggesting an association of primary hypertension with autoantibodies directed against alpha-1-adrenergic rather than beta-adrenergic receptors (18,19). First, the immunoreactive (IgG) antibodies from the VHD and HHD collectives (determined by ELISA and dot-blotting) were randomly directed with a similar frequency against all the three domains tested and both beta-AR subtypes. This is in clear contrast to our previous results obtained for a DCM collective with a high prevalence of functionally relevant anti-beta-AR (34% antibody-positive sera, 83% directed against the second extracellular receptor domain with a 86% recognition of the beta-1-AR [4]). Second, of all the VHD and HHD patients with IgG antibodies able to interact with synthetic receptor fragments, only one (VHD) patient remained who according to the previously defined criteria (4) had circulating, functionally active anti-beta-1-AR (again targeting beta-1-ECII). Third, because this VHD patient at the same time suffered from significant coronary heart disease, it seems likely that the autoantibodies have to be ascribed to concomitant ICM rather than to VHD. In addition, the functional effects obtained with this IgG preparation were similar to those previously reported for a subgroup of receptor autoantibody-positive patients with DCM or ICM (4), thus confirming the existence of functionally relevant anti-beta-1-AR in these two subsets of human cardiac disease. In addition, we identified one healthy subject who also had activating anti-beta-1-AR antibodies. We have not yet had the opportunity to follow this individual long term to determine whether he develops cardiomyopathy, nor do we have any data that would support an implication of genetic factors in the occurrence of functionally active autoantibodies. As a consequence, we intend a long-term follow-up of all receptor autoantibody-positive individuals so far identified to investigate whether activating anti-beta-AR might represent a suitable marker for the development and/or progression of DCM and/or ICM. Another interesting finding from this study was that one HHD patient suffering from a well-defined systemic autoimmune disorder (systemic lupus erythematosus) had circulating polyspecific IgG antibodies, which were also able to cross-react with intact native human beta-AR (immuno-fluorescence); however, these antibodies had no effect on receptor-mediated signaling (Fig. 3b, HHD).

Summary and perspective. Taken together, in a minor fraction of the sera from patients with cardiomyopathies secondary to VHD or HHD, we found IgG antibodies reacting with synthetic beta-AR peptides in ELISA. This finding is partially in agreement with previous reports from other groups (3,18,20). However, with one exception, none of these antibodies was able to specifically bind to intact native human beta-AR, and none of them affected receptor-mediated signaling. Thus, an approach solely based on ELISA seems not to be suited for detecting functionally relevant anti-beta-AR antibodies in patients (4,11). By applying more stringent screening criteria and, in particular, the use of functionally coupled recombinant human beta-AR situated in a cell membrane, the patient subgroups analyzed here showed no significant extent of anti-beta-AR autoantibodies. This indicates that autoimmune reactions directed against the beta-AR are unlikely to be associated with cardiomyopathies secondary to VHD or HHD.

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