Detection of Anti β1-AR Auto-Antibodies in Heart Failure by a Cell-Based Competition ELISA

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ABSTRACT

Rationale: Autoantibodies directed against the second extracellular loop of the cardiac beta1-adrenergic receptor (β1-AR) are thought to contribute to the pathogenesis of dilated cardiomyopathy (DCM) and Chagas’ heart disease. Various approaches have been employed to detect such autoantibodies; however, the reported prevalence varies largely depending on the utilized detection method.

Objective: We analysed sera from 167 DCM patients (ejection fraction < 45%), and from 110 age-matched volunteers, who did not report any heart disease themselves, with an often employed simple peptide-ELISA approach, and compared it to a novel whole cell-based ELISA using cells expressing the full transgene for the human β1-AR. Additionally, 35 patients with hypertensive heart disease (HHD) with preserved ejection fraction were investigated.

Methods and Results: The novel assay was designed according to the currently most reliable anti-TSH receptor antibody-ELISA used to diagnose Graves’ disease (“third generation assay”), and also detects the target antibodies by competition with a specific monoclonal anti-β1AR antibody (β1-AR MAb) directed against the functionally relevant β1AR epitope. Anti-β1-AR antibodies were detected in ~60% of DCM patients and in ~8 % of healthy volunteers using the same cut-off values. The prevalence of these antibodies was 17% in patients with HHD. Anti-β1-AR antibody titers (defined as inhibition of β1-AR MAb-binding) were no longer detected after depleting sera from IgG antibodies by protein G adsorption. In contrast, a previously used ELISA conducted with a linear 26-meric peptide derived from the second extracellular β1-AR loop yielded a high number of false positive results precluding any specific identification of DCM patients.

Conclusions: We established a simple and efficient screening assay detecting disease-relevant β1-AR autoantibodies in patient sera yielding a high reproducibility also in high throughput screening. The assay was validated according to “good laboratory practice” (GLP), and can serve as a companion bio-diagnostic assay for the development and evaluation of antibody-directed therapies in antibody-positive heart failure.

Keywords: heart failure, β-adrenergic receptor, auto-antibodies, ELISA, adrenergic receptors, cardiomyopathy

Non-standard Abbreviations:

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>β1-AR</td>
<td>Beta1 adrenergic receptor</td>
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<tr>
<td>β1-AR ECII</td>
<td>Second extracellular loop of the beta1 adrenergic receptor</td>
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<td>anti β1-AR antibodies</td>
<td>Antibodies, which bind to the second extracellular loop of β1-AR</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>KD</td>
<td>Affinity constant</td>
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<tr>
<td>EC50</td>
<td>Concentration which induces half maximal response</td>
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<tr>
<td>mAb 23-6-7</td>
<td>Mouse monoclonal antibody directed against the second extracellular domain of the β1-adrenergic receptor</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline, pH 7.4 (Biochrom AG, Berlin)</td>
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<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline, pH 7.4 (Biochrom AG, Berlin)</td>
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<tr>
<td>POD</td>
<td>Peroxidase</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SF9</td>
<td>Spodoptera frugiperda 9</td>
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<tr>
<td>PFA</td>
<td>Para-Formaldehyde</td>
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<tr>
<td>TMB</td>
<td>Tetramethylbenzidin</td>
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INTRODUCTION

Evidence has accumulated from both animal and patient-based studies that auto-antibodies targeting the human β1-AR may play an important role in the development and clinical course of progressive cardiac dilatation and failure (1-6).

β1-ARs are G protein-coupled receptors that trigger signalling via adenylate cyclase, cyclic adenosine monophosphate (cAMP), and PKA. This signalling pathway regulates the sarcoplasmic calcium concentration and increases cardiomyocyte contractility.

During recent years, it has been demonstrated independently by various groups that a relevant subset of these autoantibodies bind to the second extracellular loop of the β1-AR and recognize a native receptor conformation (3, 4,7,8). Such conformational anti-β1AR-(ECII) antibodies have been shown to be functionally active and may be capable of stimulating intracellular cAMP production (3, 5). Moreover, only those anti-β1AR autoantibodies that target the second extracellular loop (β1-AR-ECII) appear to be functionally active. In contrast, antibodies directed against the amino- or carboxy terminus of the receptor protein exert no biological effects (1,8,9). The presence of such functionally active, receptor-stimulating anti-β1AR-ECII is associated with a markedly worse prognosis in DCM (10).

Autoantibodies directed against the β1AR-ECII are found in 30%-75% of patients with dilated cardiomyopathy (DCM), depending on the respective study or screening method used (3,6, 10-11). In contrast, a low prevalence of these functionally active anti-β1AR-ECII has been observed in healthy individuals, or in patients who suffered from heart failure due to valvular or hypertensive heart disease (5). Recently, direct evidence has been obtained in a human-homologous rat model of heart failure, that anti-β1AR-ECII may cause a cardiac phenotype resembling human DCM (6).

The functionally relevant (conformational) epitope within the β1AR-ECII has been identified experimentally, characterized, and epitope-specific monoclonal antibodies have been generated. One of the monoclonal antibody clones, termed 23-6-7, exerted a strong stimulatory effect on β1-AR, thus mimicking the characteristics of assumingly disease-relevant anti-β1-AR autoantibodies in DCM patients.

Using a competition approach against β1IMab 23-6-7, we established a novel whole cell ELISA which specifically detects clinically relevant anti-β1AR autoantibodies in patient sera. Upon comparison of the results obtained with sera from DCM patients versus those obtained with sera from a healthy control population, the specificity of this “third generation” competition ELISA is markedly superior to the often used simple peptide-based ELISA approach.
METHODS AND MATERIALS

**Cell-based ELISA.**

Sf9 cells were grown in adhesion culture according to standard cell culture protocols. Cells were detached from culture flasks after 3-4 days of growth, when they had reached about 70-100% confluence. Afterwards, they were centrifuged (400 x g, 5 min) and resuspended in cell culture medium. Suspended cells were infected with baculovirus (MOI 6), carrying the gene for the human β1-AR. A transgene-free baculovirus served as control. Cell suspension was directly seeded on poly-L-lysine coated 96 well cell culture plates (Biocoat, # 356516) at a density of 30,000 cells per well. After 72 h incubation, half of the cell culture supernatant (200 µl/well) was removed and 100 µl 2x PFA fixation solution (2 % PFA in the final solution) was added. Cells were incubated for 15 min at RT at constant shaking. Supernatants were removed subsequently and fixed cells were washed three times with PBS (PBS Dulbecco (Cat No. L1820, Biochrom AG) + 0.1 % Tween 20 (PBS-T). Optionally the microtiter plates were frozen at -80°C for up to 6 months.

The PFA-fixed cells were blocked with 200 µl PBS-T + 3% milk powder for 1 h at RT. Afterwards, the plates were washed three times with PBS-T. Mouse monoclonal anti β1-AR antibody (23-6-7 Biogenes) was added, then 23-6-7 binding was competed by addition of human sera from healthy volunteers or from DCM patients, respectively. Positive control samples were provided by defined concentrations of monoclonal rat anti-β1-AR antibodies, which were also used for competition. After incubation for 2 h at RT with constant shaking, the cells were washed three times with PBS-T and secondary antibody solution (1:5000 in PBS-T + 3% milk powder) was added. Plates were incubated for 1 h at RT. After a further washing step, 3x with PBS-T, peroxidase bound in the complex was visualized by TMB substrate solution. After stopping the enzymatic reaction with sulfuric acid, the intensity of the resulting colour was determined at 450 nm, and at a reference wavelength of 595 nm.

No reduction in OD value of the (23-6-7) mouse antibody resulted in 0 % inhibition, whereas complete OD value reduction corresponded to 100 % inhibition.

The assay validation was conducted for the determination of the factor (K) and assay cut-off value. In three independent experiments based on the analysis of sera from 20 healthy volunteers, the factor (K) = 0.143 was obtained by using equation (1, 2, 3).

\[
\text{Inhibition\% cut-off } = \text{mean Inhibition\%row data (control samples)} + 2\times\text{SD} \quad (1)
\]

\[
K_i = \frac{(\text{Inhibition\% screening cut-off }_i - \text{mean Inhibition\%NC }_i)}{\text{mean Inhibition\%PC }_i} \quad (2)
\]

\[
K = \frac{(K_1 + K_2 + K_3)}{3} \quad (3)
\]

\[
K_i (i = 1 \text{ to } 3) \text{ was determined on three plates with 20 blank individual samples.}
\]

For all further plates “i” the following cut-off formula (4) was applied:

\[
\text{Inhibition\% cut-off }_i = \text{mean Inhibition\%NC }_i + K \times (0.143) \times \text{mean Inhibition\%PC }_i \quad (4)
\]

This way of Inhibition\% cut-off calculation avoided the necessity to analyze a high number of individual blank samples on each plate. In order to adjust the Inhibition\%row data (sample) from different plates, the respective Inhibition\% cut-off has to be considered.

\[
\text{Inhibition\%} = \text{mean Inhibition\%row data (sample)} - \text{Inhibition\% cut-off} \quad (5)
\]
Radioligand binding.
Baculo-virus-infected cells (multiplicity of infection, moi of 0.02 to 6 active viral particles/cell) were grown in large-size bottles, and harvested by scraping off with a cell wiper. Membranes were prepared by centrifugation and resuspended in TRIS-HCl (50 mmol/L, pH7.4). Radioligand binding was performed by adding iodo-cyano-pindolol (I-CYP) at concentrations of 5 pmol/L to 3760 pmol/L for 90 min at 32°C. Non-specific binding was determined in the presence of bisoprolol (1 µmol/L each). Incubation samples were filtered by GF/F-Filters (Millipore APFF #02500). Filters were washed four times with 50ml Tris-HCl, pH7.4 and counted.

ELISA based on a 26-meric peptide.
Nunc microtiter maxisorp plates were coated with 0.5µg/ml peptide, 26meric peptide HWWRAESDEARRCYNDPKCCDFVTNR, in 0,1M Na₂CO₃ or buffer alone for 16h at 4°C. After saturation of the wells with PBS supplemented with 3 % milk powder and 0,1% Tween 20, human serum from healthy volunteers or from patients with DCM, respectively, were diluted 1:20 in PBS-T +3 % BSA + 10% FCS and added to the wells. After incubation for 2 h at RT, the bound antibodies were detected by a secondary anti-human IgG antibody labelled with peroxidase, diluted 1:20000 in PBS-T + 3% milk. Between each step, plates were washed 3x with PBS-T. Afterwards, 100 µl of TMB substrate solution were dispensed to all wells. The plate was covered and incubated for 10-30 minutes at RT. The enzyme reaction was stopped by addition of 100 µL stop solution to all wells. The absorbance was read at 450 nm (reference filter 650 nm). The reduction of colour intensity was directly related to the amount of human anti-β receptor antibodies in the sample. Strong positivity was defined as 1,5 times the background density.

Patients.
167 patients suffering from dilated cardiomyopathy (DCM) were included who had been investigated by echocardiography, and their left ventricular ejection fraction had been determined to be < 45%. Sera from strictly age-matched subjects (n=110) from a local blood donor bank served as controls; all these subjects had not reported cardiovascular disease upon blood sampling. In addition, another group of patients with hypertensive heart disease was included, who had also been investigated by echocardiography, and their left ventricular ejection fraction had been determined to be > 60 %.
The mean patient age was 60.9±13.0 years in the DCM group, and 59.3±11.2 years in the volunteer group which did not differ significantly (two-tailed t-test). The mean age of the patients with hypertensive heart disease was 72±7.8 years.

Antibodies.
Large-scale production of the mouse monoclonal antibody 23-6-7, batch KD-250208-001 was performed commercially (Biogenes GmbH, Berlin, Germany). To this end, 8-wk old BALB/c female mice were immunized subcutaneously over a period of 39 days with GST fusion protein linked to a 25-meric peptide, corresponding to the β1-AR-ECII. The antibodies produced from this hybridoma cell clone were purified by Protein A affinity chromatography and dissolved in PBS. The rat monoclonal antibody clone 13F6 was produced by InVivo Biotech Services GmbH, and the hybridoma cells were generated in our own hands, using the identical GST fusion protein as the one used for mouse monoclonal antibodies. Rat antibodies were purified by Protein G affinity chromatography and dissolved in PBS. Goat polyclonal antibodies were generated by Biogenes GmbH, Berlin. The immunisation of the goats was carried out by six boosts over a period of 133 days by using the same GST fusion protein linked to the 25-meric β1AR-EC II peptide. At day 161, the antibody-containing serum was obtained and purified by affinity chromatography using a 25-meric β1AR-EC II peptide coupled to CNBr-activated Sepharose 4B (GE Healthcare, cat. 17-0430-01).

Deposit of mouse monoclonal antibody 23-6-7 and rat monoclonal antibody 13F6.
Hybridoma cells expressing antibodies 23-6-7 and 13F6 were submitted to DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig). Depositing was carried
out according to the rules of the Budapest Treaty (accession numbers DSM ACC3121 and ACC3174, respectively).

**Immunoprecipitation.**
Immunoprecipitation was performed with sera from 20 β1-AR antibody positive DCM patients. 200µl Sera were incubated with 100µl Protein G Sepharose (GE Healthcare 17-0618-05) under shaking for 16 h at 20°C. After centrifugation of the suspension in an empty column (Pierce Micro-Spin #89879) with 500g for 10min at 20°C the flow-throughs were collected.

**Data analysis.**
IC₅₀ values and sample concentration were calculated by using standard curve analysis ('four parameter logistic') from Sigma plot software, version 11. All other calculations were performed with EXCEL software, version 2003/2007.

## RESULTS

*Novel cell-based ELISA using the fully recombinant human β1AR.*

In order to provide the native and functionally active β1-AR as binding target for auto-antibodies, SF9 cells were infected with a baculovirus carrying the transgene for the human β1-AR and coated on 96 well plates. Direct measurement of patients’ anti-β-AR auto-antibody titers by a simple ELISA approach using infected SF9 cells was not feasible due to a strong background signal. This non-specific background was probably due to recognition of other cell surface epitopes by the highly diversified human antibody pool. In order to circumvent this problem, a competition assay was established, in which a high affinity antibody directed against the β1-AR-EC II was used to generate a specific binding signal at the β1-AR-expressing SF9 cells, which was then specifically competed by the anti-β1-AR auto-antibodies directed against the same epitope present in patient sera (Figure 1).

*Determination of the density and affinity of recombinant human β1ARs.*

Infected SF9 cells were harvested, and the density and affinity of recombinant receptors was determined by radioligand binding. Whereas only a small non-specific signal was determined in non-infected or control virus-infected cells, the density was 219406 ± 34456 receptors/cell after infection at a multiplicity of infection (moi) of 0.02 active virus particles per cell, and amounted to 246221 ± 95436 receptors/cell after infection with 0.2 active virus particles per cell, reaching a plateau with higher moi values (350376 ± 30048 receptors/cell after infection with 2 active virus particles per cell, and 330990 ± 41170 receptors/cell after infection with 6 active virus particles per cell). The respective binding affinities of the radioligand (kd values) were 36, 37, 24 and 32 pmol/L, respectively, and did not vary significantly between the groups.

Also the affinity of the allosteric binding site for stimulatory anti-β1-AR antibodies were tested in SF9 cells which had been infected with mois ranging from 0.2 to 6 active virus particles per cell. In these cells, EC50 values of ELISA binding assays were analysed using the antibody 23-6-7 (as depicted in Figures 2), and kd values were calculated. The kd values ranged between 0.22 and 0.43 nmol/L, and did not differ significantly between the groups.

The kd values did not differ significantly when various passages (23 to 51 passages) of SF9 cells were used and compared in these experiments.

*Identification and characterisation of the monoclonal anti-β1-AR antibody clone 23-6-7.*

A prerequisite for such a competition approach was the generation of an antibody with high specificity and affinity to β1-AR. Various monoclonal mouse antibodies to β1-AR ECII were

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produced by using a hybridoma cell line-based approach, and clone 23-6-7 was identified as the most promising monoclonal antibody.

In order to prove the binding specificity, we incubated various concentrations of 23-6-7 on recombinant β1-AR-overexpressing SF9 cells and initially measured its binding characteristics in the absence of any competitor. The results are shown in Figure 2 which illustrates an IC50 value of 0.43 nM. To determine the functionality of the 23-6-7 anti-β1-AR antibody clone, we investigated its ability to activate receptor-mediated intracellular cyclic adenosine monophosphate (cAMP) accumulation through sequential activation of Gs proteins and adenylyl cyclase (AC). One method to detect this increase in intracellular cAMP is to use fluorescence resonance energy transfer (FRET) between cyan fluorescence proteins (CFP) and yellow fluorescent proteins (YFP) fused to the cAMP-binding domain of Epac1 (10). Addition of clone 23-6-7 clearly activated HEK 293 cells stably expressing human β1-AR, as determined by using this FRET assay (Figure 3B), with slow kinetics as are typically exerted by anti-β1-AR auto-antibodies from DCM patients (Figure 3C). In contrast, a negative control antibody was ineffective (Figure 3A).

Cell-based β1AR competition ELISA.

Our hypothesis was that the binding of the monoclonal anti-β1-AR antibody 23-6-7 to β1-AR-overexpressing SF9 cells should be modified by co-incubation with human DCM patient serum (schematic overview in Figure 1). A competitive reduction of the 23-6-7 antibody binding should occur, depending on the presence of anti-beta1-AR antibodies in the respective sample.

As a first step to establish such an assay, polyclonal goat anti-β1-AR antibodies were spiked to serum pool derived from healthy volunteers to mimic the presence of human anti-β1-AR antibodies. To clarify the impact of adding this human serum pool to the assay, the polyclonal goat anti-β1-AR antibodies were also added in control buffer in an identical assay approach. Figure 4 shows high similarity of the competition curve for both conditions, regarding both, dose-dependency and maximum signal. In order to validate the assay, a negative control, consisting of a serum pool from healthy volunteers, was deemed to be necessary. Assay sensitivity was determined at 10 nM when using the polyclonal goat anti-β1-AR antibody for competition.

Validation of the β1-AR ELISA.

To warrant inter-assay comparability, a negative control sample (NC), consisting of pooled human serum samples from healthy volunteers and a positive control (PC), consisting of a human serum pool spiked with rat anti-β1-AR antibodies (13F6) were measured on each microtiter plate. We used a monoclonal rat (13F6) antibody instead of the polyclonal goat anti-β1-AR antibody because of its more reproducible availability.

In order to classify the inhibition (%) of the human serum samples, the plate specific Inhibition\% cut-off was considered. Responses varied between individual assays – therefore, cut-off values were modified accordingly. The use of the negative control plus a predetermined factor (K) to assess the cut-off value in each assay allowed to correct for changes of the non specific binding (NSB) over time. The additional use of the positive control in the cut-off formula allowed for an even better normalization, because only the OD value of the positive control allows an assessment of assay sensitivity.

Assay cut-off point value: The cut-off value was determined statistically based on the level of non-specific background of the assay and the response of those matrix samples, above which a positive response was detected. In three independent experiments, serum samples from 20 healthy volunteers were examined. The mean \( + 2.0 \times \text{SD} \) was calculated to determine the cut-off. In order to account for some smaller variation between individual assays, an adjusted cut-off value was calculated by multiplying with a specific normalization factor, determined from the pre-study validation data.

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**Sensitivity:** Assay sensitivity was determined as the concentration at which the antibody preparation produced an assay readout equal to the cut-off value. Because it was so far not possible to purify human anti-β1-AR antibodies sufficiently from patient sera, the assay sensitivity was determined by using the polyclonal goat anti-β1-AR antibody, as described before. The cut-off value was determined at approximately 10 nM.

**Recovery:** To determine recovery, 20 plasma samples from healthy volunteers were spiked with rat 13F6 anti β1-AR antibodies (assay concentration: 760 nM). All 20 samples showed inhibition values above the cut-off point value with mean coefficients of variation (CV) of 2.54 % and therefore completely fulfilled the criteria for recovery.

**Precision:** Intra-assay (repeatability) and inter-assay (intermediate precision) variability was evaluated by using a validation sample (VS) and a positive control (PC) both spiked with rat 13F6 antibodies at an assay concentration of 253 nM and 760 nM respectively. Four replicates were used on each plate, which were carried out on three different days. We found a mean intra-assay CV of 4.8% and an inter-assay CV of 16.2% for the VS, and a mean intra-assay CV of 3.6% and an inter-assay CV of 15.4% for the PC, respectively.

Measurement of the 167 human DCM serum samples and 110 age-matched volunteers in three independent measurements resulted in a mean inter-assay CV of 14.4% for the patient group, and of 16.9% for the control group.

**Stability:** Storage conditions and blood serum sample stability was investigated for the VS. Storage at either 22°C for 3h or at 4°C for 16 h had no negative impact on the measurement of rat 13F6 anti β1-AR antibodies and resulted in 95.1% and 92.3% recovery compared to the unstressed VS. Also three times repeated freeze/thaw cycles had no influence on the results of the VS.

Additionally, the stability of anti-β1-AR antibody determination was analysed in whole blood samples. Ten DCM samples, which were tested positive for anti-β1-AR antibodies, were stored at 20°C for 20h and analysed again. A recovery of 94.7 % (SD ± 10.4) was determined, thus showing a high antibody stability in whole blood comparable to the stability in serum.

**Screening results of DCM vs. volunteers, and HHD.**

We evaluated the presence of anti-β1-AR antibodies in 167 DCM patients presenting with an LV-EF < 45% and of 110 age-matched volunteers who reported no known heart disease upon blood sampling. In the DCM group, we identified 62.2% of these samples to be positive for relevant anti-β1-AR antibodies, and only 8.2% in the age-matched control group (Figure 5A). In a second consecutive study, we also investigated 35 patients with hypertensive heart disease (HHD) and preserved LV ejection fraction. In contrast, in the somewhat older HHD patient population, we identified 17 % of these samples to be positive (Figure 5C).

In order to demonstrate that the inhibition values which were determined in the cellular ELISA were actually due to antibodies, 20 anti-β1-AR antibody-positive DCM sera were depleted via Protein G Sepharose to eliminate IgG immunoglobulins. The flow-through from each serum sample was collected and analysed in comparison to the load (untreated serum) by cellular ELISA. We observed that ELISA-determined anti-β1AR titers disappeared completely in all investigated antibody-depleted samples (nominal mean Inhibition % was reduced from 13.1 % to -31.1%, Figure 6).

**Peptide-based ELISA.**

A widely used method for determination of anti-β1 receptor antibodies in human serum is a peptide-based ELISA, in which a 26-meric peptide (HWWRAESDEARRCYNDPK CCDFVTR), corresponding to the amino acid sequence (residues 197-222) of the second extracellular loop of the
human β1 receptor, is immobilized on plastic surfaces in order to capture specific human anti-β1 receptor antibodies. We conducted the same method to clarify the potential as a diagnostic tool for this ELISA assay.

Identical serum samples from DCM and age-matched volunteers were analysed as used in the cellular SF9 β1-AR ELISA assays.

We observed 29.9% anti-β1-AR antibody positive DCM patients versus 35.5% positive findings in the control group (Figure 5B).

DISCUSSION

Autoantibodies directed against β1-adrenergic receptors seem to play an important role in the pathogenesis of heart failure, particularly in the pathogenesis of dilated cardiomyopathy (DCM) and Chagas’ heart disease (1,3,6,8,9). Putative mechanisms by which anti-β1-AR autoantibodies exert their adverse effects on cardiomyocytes are complex and have been intensively investigated over the past two decades.

Here we report the establishment of a novel cell-based competitive ELISA for the detection of functionally active human anti-β1-AR autoantibodies. The assay uses the fully native β1-AR protein as target antigen to provide a correct folding of the extracellular domains which is a basic requirement to identify epitope-specific autoantibodies. In order to optimize the specificity of the assay, a competitive approach was developed using the monoclonal anti-β1-AR antibody 23-6-7. This antibody binds to the second extracellular loop of the human β1-AR and is able to stimulate receptor activity. Functionally relevant human anti-β1-AR autoantibodies from patient sera are characterized by their capacity to bind to the same or overlapping epitopes and displace this monoclonal antibody and therefore reduce the ELISA signal. An epitope search by alanine permutation scanning has yielded hints that within the EC II loop of the β1-AR, the amino acid sequence NDPK should be part of the relevant epitope.

As opposed to classical β1AR agonists such as catecholamines, which bind to the typical receptor binding pocket, the agonistic anti-β1-AR autoantibodies bind to the second extracellular loop of the receptor molecule. This atypical binding site is characterized by allosteric binding kinetics, in which no high or low affinity components can be distinguished. This finding was reconfirmed in our experiments which documented a single-phase binding plot of the agonistic monoclonal antibody 23-6-7 (please see Figure 2). The absolute receptor densities were counterchecked by radioligand binding, which identified a multiplicity of infection (moi) of 2 active viral particles as sufficient to generate a maximum receptor density on infected sf9 cells. The assay was best reproducible at a moi of 6 units, which was therefore used for establishing the assay.

Previous studies used several other screening methods for detecting anti-β1-AR autoantibodies. Autoantibodies against the second extracellular loop of the β1-AR were found in 30%-75% of DCM patients in these previous studies (1,3,6). The inconsistency among studies may be due to the lack of standardized measurement methods. The definition of antibody positivity depended on divergent screening methods. These assay approaches can be divided into two classes:

1) assays which investigate the functional capacity of the antibodies to activate β1AR
2) assays which analyse the binding characteristics of the auto-antibodies to the ECII loop of the β1-AR.

Functional assays, which measured e.g. contractility effects on neonatal rat cardiomyocytes or chick embryo hearts, or β1-AR-mediated cAMP accumulation, were established and adapted to detect functional anti β1-AR antibodies (5,6,13-15). All these functional assays are characterized by
procedures which are time- and cost-consuming, or difficult to standardize, and cannot be reasonably used to screen larger patient populations (n >1000) rapidly.

Binding of human anti-β1-AR autoantibodies was mostly investigated by using peptide-based ELISAs. To this end, a linear 26-meric peptide, which corresponds to the 2nd extracellular loop (amino acid position 197-222) of the human β1-AR, was immobilized onto microtiter plates (16,17). This kind of assay is fully HTS (high throughput screening) adapted, but its use as a screening assay with diagnostic relevance had not yet been simultaneously investigated in a larger population of patients and age-matched controls who did not report any known heart disease.

The present study represents a new approach to systematically compare these populations on a strictly age-matched basis. Another previous publication had investigated healthy subjects using the same 26-meric β1-AR-EC II-peptide-based ELISA (18), and found 10% false positives, but the mean age of that control group was markedly lower (36.6 years) than that of the investigated DCM patient group (55.7 years). These authors (18) as well as many other researchers (19) have found that the number of false positive biomarker results markedly increases with increasing age of the investigated control population, and this also corresponds to our own experience with several anti-β1-AR antibody ELISA assays, so that we would think that our study has the advantage to strictly control for this important parameter. In contrast, in another, even older patient population with HHD, but preserved LV ejection fraction, we found a somewhat increased prevalence compared to the control group. This low prevalence for patients who did not present with clinically overt heart failure compares to the results of previous studies (5).

Another interesting question is the prevalence of autoantibodies in patients with heart failure due to severe coronary artery disease (“ischemic cardiomyopathy”, ICM). This question should be investigated in future studies.

The novel whole cell-based competition ELISA yielded a markedly lower rate of false positives compared to peptide-based assay. We tested the same DCM and age-matched control population in a simple 26-meric binding assay. We found that 29.9% of DCM patients and 35.5% of age-matched volunteers were tested positive. The result for the DCM group is within the range of data from other studies which investigated anti-β1-AR antibodies by using peptide-based ELISAs (16,20,21). We suppose that some of the positive findings obtained in the control group are due to cases of existing but yet undiagnosed heart failure, since the prevalence of undiagnosed heart failure with impaired cardiac LV ejection fraction (EF < 35%) is about 7.7%; 4% of randomly selected asymptomatic men over 40 years even had an EF < 30% (22). The health status in the volunteers of the present study was checked by a self-reporting questionnaire, an echocardiographic recording of LV function was not feasible for the whole population. Additionally, a number of the results in this group are very probably due to false positive sera: this percentage seems to be in the lower single-digit range for the novel assay. In contrast, it amounted to about 26% (31% minus about 5% assumed cases of yet undiagnosed heart failure) in the peptide-based assay. Thus, the percentage of false positives obtained with the novel cell-based competition ELISA compares quite well to what is found with even the most advanced assays for anti-TSH antibodies in Graves’ disease (23), whereas it seems to be rather too high for the peptide-based assay.

In order to verify the assay specification we conducted a validation procedure according to “good laboratory practice” (GLP). Besides the determination of the cut-off values, the precision and reproducibility were verified in detail. In summary, the assay shows high accuracy (intra-assay comparison as well as in the inter-assay assessment) with CV values < 20% for positive control and validation samples.

The novel approach to use a monoclonal antibody which competes with human anti-β1-AR antibodies for immobilized native β1-ARs was conceived very much in parallel to a widely distributed ELISA which measures functionally active, stimulating auto-antibodies against the thyroid stimulating hormone (TSH) receptor in Graves’ disease. This so called 3rd generation ELISA has shown the

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highest sensitivity and robustness, and is now commonly used as a gold standard diagnostic assay (24,25). The ELISA uses the specific activating monoclonal anti-TSHR antibody M22 and the fully human TSH receptor protein expressed on recombinant cells, and has largely replaced the previously used radioimmuno assay (RIA) which was based on a competition for the natural ligand TSH (26). A prerequisite for both, RIA and ELISA was the use of the native fully expressed receptor, which provided a conformationally active epitope.

In contrast, several attempts to establish large scale ELISAs based on peptides from the TSH receptor failed. Also approaches which assessed the direct binding of human serum samples to the recombinant TSH receptor protein turned out to be unreliable (27). These findings are very much in line to our results on anti-β1-AR antibodies.

Similarly, auto-antibodies against the acetylcholine receptor which occur in myasthenia gravis are most reliably determined by an assay which uses the full alpha-bungarotoxin-labelled receptor protein as an antigen (28).

The novel assay holds promise to identify especially well those DCM patients who should benefit most from removal of anti-β1-AR autoantibodies. To this end, several therapeutic approaches have been developed: Several clinical trials with DCM patients showed that elimination of anti-β1-AR antibodies by immunabsorption (IA) led to an increase in several cardiac functional parameters, followed by an improvement of the NYHA functional class (29). Most notably these studies found a significant improvement in LV ejection fraction (LVEF) for both non-selective antibody removal and selective anti-β1-AR antibody removal using β1AR-peptide-coated columns (29-31), inferring that anti-β1-AR antibodies are the disease-relevant antibodies.

Another approach to lower cardiac auto-antibodies is the use of a peptide to induce antigen-specific tolerance and to reduce the response of an overactive immune system, which is currently in clinical development (32).

Both treatment strategies share the need of a reliable diagnostic assay to screen for anti-β1AR autoantibodies and thus to reliably identify antibody-positive heart failure patients. It was the scope of the present study to define the experimental basis for future clinical studies, which will have to include larger patient numbers to finally assess the relevance of this parameter as a prognostic marker in heart failure.

CONCLUSION

We have established a simple and efficient screening assay which identifies relevant auto-antibodies directed against the β1-AR in human sera. These autoantibodies can be detected and quantified with high reproducibility in a high throughput screening approach. The assay was validated according to “good laboratory practice” (GLP), and now serves as a companion bio-diagnostic assay to develop and control individualized therapies in antibody-positive patients. It can be easily and reproducibly established in any laboratory in the world. Future modifications could include the use of permanently β1-AR-overexpressing cell lines, or of membrane preparations of β1-AR-overexpressing cells.

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DISCLOSURE STATEMENT
Authors Münch, Boivin-Jahns, Holthoff, Zeibig, Bauer, Lohse, Jahns were either employed by, or held stake of the biotech Company Corimmun GmbH, Martinsried, Germany. Authors Kääb and Clauss and have no interests to disclose.

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FIGURE LEGENDS

**Figure 1**: Principle of the ELISA measurement of human anti-beta1-AR antibodies via competition of the anti-beta1-AR monoclonal antibody 23-6-7. The ELISA mimics the in vivo auto-antibody binding characteristics to β1-ARs using a microstate plate format. In order to avoid cross-binding of other human antibodies to various cellular membrane proteins, a competitive approach was developed: specific human anti-β1-AR antibodies compete with the monoclonal antibody 23-6-7 for the binding to cellular β1-ARs.

**Figure 2**: Binding affinity of the monoclonal antibody 23-6-7 to fully recombinant human β1-AR, overexpressed on SF9 cells. Means with S.E.M. of at least 4 independent measurements are plotted.

**Figure 3**: Measurement of cAMP levels by Epac-FRET in human embryonic kidney HEK293 cells stably expressing human β1-ARs. Representative FRET ratio traces of independent experiments are presented (% corresponds to the relative change in YFP/CFP intensity ratio). The decrease in FRET reflects an increase in intracellular cAMP. (A) None of the inactive control antibodies induced a significant cAMP response in living cells. The viability of the cell is proven by additional stimulation by isoproterenol (Iso) at a concentration of 2.5 mol/L at the end of the experiment, which elicits a full FRET response. (B) In contrast, addition of antibody 23-6-7 elicited a relevant signal, which corresponds to 38.2 % of the maximum possible signal, as was induced by additional administration of Iso at the end of this experiment. (C) The signal intensity and kinetics were comparable to those from DCM patient sera previously judged anti-β1-AR antibody positive.

**Figure 4**: Competition of the binding of the mAb 23-6-7 by polyclonal goat anti-β1-AR antibodies. Various concentrations of polyclonal goat antibodies were co-incubated with the mAb 23-6-7 at a final concentration of 0.26 nM. Addition of 10% serum pool derived from healthy volunteers was compared to buffer control and resulted in a similar dose-dependent effect. Inhibition was exerted by at least 10 nM goat antibodies. Means with S.E.M. of at least 4 independent measurements are plotted.

**Figure 5**: A. Overview of the β1-AR binding activity of DCM patients (n = 167) and control subjects who did not report any known heart disease (n= 110) using an ELISA with SF9 cells overexpressing β1-AR vs. control SF9 cells (negative for β1-AR). The binding activity was determined by measuring the competition with the monoclonal anti-β1-AR antibody 23-6-7. Means with S.E.M. of 3 independent measurements are plotted. B. Identical serum samples were analysed against the 26mer peptide. Binding activity was calculated as a ratio (sample OD to 26mer / sample OD to control well). An anti-β1-AR antibody positive score was defined as a ratio of > 1.5. Means with S.E.M. of 2 independent measurements are plotted. C. Overview of the β1-AR binding activity of HHD patients (n = 35) using an ELISA with SF9 cells overexpressing β1-AR vs. control SF9 cells (negative for β1-AR).

**Figure 6**: Comparison of the inhibitory effect of unaltered sera and the respective antibody-depleted serum fractions. The 20 original serum samples were tested positive with a mean inhibition value of 13.1%. In contrast, all protein G- treated samples were tested negative with a mean inhibition value below cut-off value.

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Novelty and Significance

What Is Known?

- Autoantibodies against β1-adrenergic receptors (β1-ARs) may contribute to heart failure, such as dilated cardiomyopathy (DCM) and Chagas heart disease.
- Several diagnostic ELISA assays have been used, mostly based on coated peptides.

What New Information Does This Article Contribute?

- A new assay was designed in analogy to the anti-TSH receptor antibody ELISA (Graves’ disease).
- The full-length β1AR protein is used as the binding target.
- β1-AR auto-antibodies are detected by competition with a specific monoclonal antibody directed against the functionally relevant epitope.
- The assay had high reproducibility and CV values comparable to other standard assays.
- The ELISA can serve as companion bio-diagnostic assay for the development of novel therapeutic approaches.

Autoantibodies directed against the second extracellular loop of the β1-AR are thought to contribute to the pathogenesis of DCM and Chagas heart disease. We analysed sera from DCM patients and from age-matched volunteers with an often used peptide-ELISA approach, and compared it to a novel whole cell-based ELISA using cells expressing the full transgene for the human β1-AR. This assay was designed according to the most reliable anti-TSH receptor antibody ELISA used to diagnose Graves disease (“third generation assay”). It detects the target antibodies by competition with a specific monoclonal antibody directed against the functionally relevant β1AR epitope.

Anti-β1-AR antibodies were detected in ~60% of DCM patients and in ~8 % of healthy volunteers. The novel assay yielded a high reproducibility in high throughput screening, and a low number of false positives. These features render it superior to the existing assays. The assay was validated according to “good laboratory practice” (GLP), and can serve as a companion bio-diagnostic assay for the development and evaluation of antibody-directed therapies in antibody-positive heart failure.
Figure 1

- Microtiter well coated with β1-AR over-expressing cells

- Anti-β1-AR antibody-positive human serum
  - Strong signal

- Anti-β1-AR antibody-negative human serum
  - Weak signal

- mAb (mouse)
- POD
- Human antibodies
- β1-AR

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Figure 2

![Graph showing binding of monoclonal anti-β1-AR antibody 23-6-7 to β1-AR (arbitrary photometric units) as a function of concentration (nmol/L).]
Figure 3

- Inactive control antibody
- Monoclonal antibody 23-6-7
- Human anti-β1-AR antibody

Graphs showing the normalized FRET ratio over time for each antibody in response to isoproterenol.
Figure 4

Inhibition (%) of mAb (23-6-7) binding to β1-AR

- buffer control
- 10% serum

concentration of polyclonal anti-β1-AR antibodies (goat) (nmol/L)
Figure 5 A

DCM (n =167)  
age-matched volunteers (n =110)  
(no self-reported heart disease)
Figure 5 B

DCM (n =167) vs age-matched volunteers (n =110)
(no self-reported heart disease)
Figure 5 C

HHD (n = 35)
Figure 6
Detection of Anti β1-AR Auto-Antibodies in Heart Failure by a Cell-Based Competition ELISA
Hans-Peter Holthoff, Stefan Zeibig, Valerie Boivin, Johannes Bauer, Martin J. Lohse, Stefan Kääb, Sebastian Clauss, Roland Jahns, Angela Schlip, Götz Münch and Martin Ungerer

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