Cardiomyopathy

Distinct Patterns of Autoantibodies Against G-Protein–Coupled Receptors in Chagas’ Cardiomyopathy and Megacolon
Their Potential Impact for Early Risk Assessment in Asymptomatic Chagas’ Patients

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Objectives
Distinguishing the patterns of autoantibodies (AAB) against G-protein–coupled receptors in Chagas’ cardiomyopathy and megacolon and the discovery of such a pattern in patients who are as yet asymptomatic could help to identify patients at high risk of developing the life-threatening complications of Chagas’ disease.

Background
Such AAB against receptors as beta 1 (beta1-AAB), beta 2 (beta2-AAB), and muscarinic 2 (M2-AAB) are thought to be involved in the pathogenesis of Chagas’ cardiomyopathy and megacolon, the predominant manifestations of Chagas’ disease, which is the most serious parasitic disease in Latin America.

Methods
Beta1-AAB, beta2-AAB, and M2-AAB were measured in the serum of asymptomatic Chagas’ patients and in those with cardiomyopathy and/or megacolon.

Results
Nearly all Chagas’ patients with cardiomyopathy and/or megacolon had AAB. Predominance of beta1-AAB combined with M2-AAB in Chagas’ cardiomyopathy and beta2-AAB with M2-AAB in megacolon was found. Such patterns were also found in 34% of the asymptomatic patients, of whom 85% possessed a beta1-AAB level typical for Chagas’ cardiomyopathy.

Conclusions
The percentage of asymptomatic Chagas’ patients who had a specific AAB pattern and had a beta1-AAB level above a defined cutoff point mirrors very well the epidemiological situation, which showed that clinical manifestations develop in nearly 30% of Chagas’ patients and cardiomyopathy in nearly 90% of them. We hypothesize that beta1-, beta2-, and M2-AAB measurement might be a useful tool for risk assessment in the indeterminate state of Chagas’ disease to select patients for earlier involvement in care programs. However, prospective studies are needed to further evaluate this hypothesis. (J Am Coll Cardiol 2010;55:463–8) © 2010 by the American College of Cardiology Foundation

Chagas’ disease, caused by Trypanosoma cruzi infection, was documented for the year 2006 to affect 15 million patients, with an annual incidence and mortality of 40,000 and 12,500 subjects, respectively, making it the most serious parasitic disease in Latin America (1). Consequently, Chagas’ disease places an enormous burden on the countries’ economic resources and dramatically affects the patients’ social and labor situation (2,3).

Despite being lifelong parasite carriers, two-thirds of the patients remain in a clinically asymptomatic (indeterminate) state, whereas clinical manifestations develop in one-third, with a latency of 10 to 30 years. In nearly 90% of these cases, the patient’s heart is affected and the patient presents with cardiomyopathy associated with arrhythmias, heart failure, and frequently sudden death. The remaining 10% have gastrointestinal disorders, mainly megacolon or megaesophagus (4–6). Unfortunately, there is no tool available for identification of patients who possess a high risk for progressing from the indeterminate state into the state associated with the life-threatening complications.
After the discovery of autoantibodies (AAB) against G-protein-coupled receptors in Chagas' cardiomyopathy and megacolon, such as those against the beta 1- and beta 2-adrenergic receptor (beta1-AAB, beta2-AAB) and the muscarinic 2 receptor (M2-AAB), which suggests an autoimmune etiology (7,8), we hypothesize that such AAB could possess the potential for risk assessment in asymptomatic Chagas’ patients. Therefore, we looked for specific AAB patterns that occur in the different organ manifestations and afterward for the occurrence of such patterns in asymptomatic patients.

Methods

Study participants. The retrospective study was approved by the authorities of the Santa Bárbara Hospital, Sucre, Bolivia. All patients signed an informed consent form. Serum was taken from 29 healthy controls (C) and, between July 2006 and August 2007, consecutively from 228 patients with Chagas’ disease. Patients were indicated by positivity for Trypanosoma cruzi detected by enzyme-linked immunosorbent assay (Wiener Laboratorios S.A.I.C., Rosario, Santa Fe, Argentina) and indirect hemagglutination (Polychaco S.A.I.C., Buenos Aires, Argentina). Patients were admitted to the cardiological and gastroenterological departments, and there they were classified by clinical investigation, electrocardiographic mapping, and radiological imaging into mild, moderate, and severe categories. The presence of at least 1 of the following criteria was used to classify the cardiomyopathy; mild: mild electrocardiographic changes in ventricular repolarization, sinus bradycardia; moderate: left anterior fascicular block, incomplete left bundle branch block, right bundle branch block, second-degree atrioventricular block Mobitz type I, atrial fibrillation in patients age >50 years; and severe: cardiomegaly, dilatative cardiomyopathy, complete left bundle branch block, atrioventricular block Mobitz type II, complete atrioventricular block, atrial fibrillation in patients age <50 years. The patients’ basic characteristics are shown in Table 1.

For identification and quantification of the AAB, a bioassay was used that was first established for measurement of AAB against G-protein-coupled receptors by Wallukat and Wollenberger (9), and is briefly described here. In this bioassay, the chronotropic response of spontaneously beating cultured neonatal rat cardiomyocytes was recorded, which is the sum of positive chronotropy by beta1- and beta2-AAB and negative chronotropy by M2-AAB (1 U of AAB activity = 1 beat/min frequency change).

To differentiate the AAB species with respect to their contribution to the chronotropic response, analysis was conducted in the presence of specific antagonists such as ICI-118.551 for beta2-AAB, atropine for M2-AAB, and propranolol for beta1-beta2-AAB. The AAB positivity was defined using cutoff values that were calculated based on x ± 3 SD of the AAB levels of more than 100 healthy subjects found in previous studies. For the positive chronotropic response of cultured neonatal rat cardiomyocytes to beta1- and beta2-AAB, a cutoff ≥4.0 U and for the

### Table 1

<table>
<thead>
<tr>
<th>Category of Cardiomyopathy (%)</th>
<th>C</th>
<th>I</th>
<th>CM</th>
<th>MC</th>
<th>CM+MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>5</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>44</td>
<td>56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe</td>
<td>51</td>
<td>29</td>
<td></td>
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</table>

Basic data and percentages of positivity for autoantibodies (AAB) against beta 1-adrenergic, beta 2-adrenergic, and muscarinic 2 receptors (beta1-AAB, beta2-AAB, M2-AAB) of the 228 Chagas’ patients and 29 healthy subjects. *CM > MC, p < 0.001.

C = healthy control subjects; CM = patients with chronic Chagas’ disease manifested as cardiomyopathy; CM+MC = patients with chronic Chagas’ disease manifested as cardiomyopathy combined with megacolon; I = patients with Chagas’ disease in the indeterminate (asymptomatic) state; MC = patients with chronic Chagas’ disease manifested as megacolon only.
negative response to M2-AAB a cutoff $\geq -4.0$ U was calculated.

**Cardiomyocyte preparation and culturing.** Hearts of 1- to 3-day-old rats were removed under sterile conditions and transferred to phosphate-buffered saline (PBS) solution containing penicillin/streptomycin. The ventricle tissue was separated, dissected in pieces, and washed twice with 10 ml PBS containing penicillin/streptomycin and at last once with PBS only. The ventricle pieces were suspended in 30 ml PBS containing 0.2% trypsin. After incubation for 20 min at 37°C, the trypsinization was stopped with 10 ml ice-cold heat-inactivated calf serum. The resulting suspension was centrifuged at 130 g for 6 min, and the pellet was transferred to 20 ml SM20-I medium. For cell count estimation, 100 $\mu$l of this suspension was added to 100 $\mu$l trypan blue solution. For cell culturing 2.4 $\times$ 10$^6$ cells were suspended in 2.5 ml of glucose containing SM20-I medium, which was equilibrated with humid air and supplemented with 10% heat-inactivated calf serum, 0.1 mU insulin, and 2 $\mu$M fluoredoxyuridine (for prevention of overgrowth of the myocytes by nonmyocytes), transferred to 12.5-cm$^2$ Falcon flasks, and cultured as monolayer for 4 days at 37°C. The medium was renewed after 2 days.

**Assay standardization.** On the fourth day, the medium was removed and replaced by 2 ml of SM20-I containing 10% heat-inactivated calf serum and incubated at 37°C for 2 h. After the incubation, the flasks were transferred onto a heated stage (37°C) of an inverted microscope where 8 synchronously beating fields of the cell layer were marked. The number of beats of each field was counted for 15 s and averaged with computer assistance using the Modular Image Analyzing System Imagequant-Fourier Analysis version 1.1 (Mediquant GmbH, Lützen, Germany). To use the bioassay for the estimation of the AAB activities, the following criteria must be fulfilled: 1) the basal beating rate must range between 120 and 160 beats/min; 2) cells stimulated for 5 min with isoprenaline (10 $\mu$M) as positive control must respond with a frequency increase of more than 45 to 50 beats/min; and 3) because in patients with Chagas’ disease beta1-, beta2-, and M2-AAB recognize epitopes on the second extracellular loops of the receptors and the antibodies were neutralized using peptides corresponding to these loops, cells must respond to a further control with a frequency increase of 20 to 30 beats/min. In patient immunoglobulin G was added (dilution 1:20) to the assay medium and incubated for 1 h. Then the beating rate of the same fields as used for the estimation of the basal rate was counted to calculate the ratio of beating frequency.

**MEASUREMENT AND DIFFERENTIATION.** In cell cultures prepared and cultured as described earlier, the basal beating rate of 8 fields of the cell layer was counted. The addition of atropine (final concentration 10 $\mu$M) served as the inhibition of the M2 receptors of the cells, excluding negative chronotropic interferences and enabling the measurement of the positive chronotropic activity of the patient AAB preparation. For this purpose, patient immunoglobulin G was added (dilution 1:20) to the assay medium and incubated for 1 h. Then the beating rate of the same fields as used for the estimation of the basal rate was counted to calculate the ratio of beating frequency, which represents the sum of the beta1- and beta2-AAB activity. To separate the beta1- from the beta2-AAB activity, the beating rate was counted in the presence of the specific beta2-antagonist ICI-118.551 (0.1 $\mu$M).

The M2-AAB activity was measured in a separate experiment. After recording the basal beating frequency, 1 $\mu$M propranolol was added, which antagonizes beta1- and beta2-AAB. Therefore, all interferences caused by positive chronotropic AAB can be excluded. Patients’ M2-AAB activity (negative chronotropic) was calculated by measuring the decrease of the beating frequency.

**Statistical analysis.** Patient age is presented as median, maximum, and minimum. The AAB levels are expressed as a box plot showing a median bar, 25th and 75th percentiles (interquartile range), whiskers with ends that represent the largest and smallest values inside the 1.5 $\times$ interquartile range, and outliers (open circles) defined as a value between the 1.5 $\times$ and 3 $\times$ interquartile range. Statistical analysis was performed using the SPSS software package (SPSS Inc., Chicago, Illinois). Correlations were checked by determination of the Spearman correlation coefficient. For dichotomous data, the chi-square and Fisher exact test were used. Intergroup comparison was analyzed by the Kruskal-Wallis H-test and as a
Comparing the AAB levels of the patients in the indeterminate state or having cardiomyopathy (groups CM and CM+MC) or megacolon only and who were positive for the AAB combinations beta1-/ or beta2-/M2-AAB, beta1- and M2-AAB were significantly higher in patients with cardiomyopathy than in the megacolon patients. In contrast, beta2-AAB was highest in the patients with megacolon only. The patients in the indeterminate state had higher beta1- and M2-AAB levels than the megacolon patients. No significant differences in the AAB levels were seen in the patients in the indeterminate state and those with cardiomyopathy (Fig. 1).

To find out which AAB level best distinguished between patients with cardiomyopathy and those with megacolon, we constructed the receiver-operator characteristic curve. For the beta1-AAB curve, all patients with cardiomyopathy (groups CM and CM+MC) versus those with megacolon only were included in the analysis. The cutoff of 8.9 U for beta1-AAB (sensitivity 0.94, specificity 0.85, positive predictive value 0.96, and negative predictive value 0.82) was found to be the best to distinguish between patients with cardiomyopathy and those with megacolon (Fig. 2).

Next we used this cutoff to determine the percentage of patients in the indeterminate state who presented with the cardiomyopathy- and megacolon-specific level. In the indeterminate patients positive for the AAB combinations beta1-/M2-AAB, beta2-/M2-AAB, or both combinations, the cardiomyopathy-specific beta1-AAB level ≥8.9 U was found in 90% of the patients, whereas 10% showed a level characteristic for megacolon.

Additionally, we show in Figure 3 an increase in the beta1-AAB level from patients in the indeterminate state thought to have risk for cardiomyopathy (beta1-/M2-AAB positivity, beta1-AAB level ≥8.9 U) to patients with mild

post-hoc analysis by the Mann-Whitney U test without correction for the multiple comparison.

**Results**

As shown in Table 1, control subjects and patients in the indeterminate state were younger than the patients with cardiomyopathy and megacolon. However, no correlation existed between patient age and AAB positivity or AAB levels.

In the control group, beta1- and M2-AAB were absent in 100% of the subjects, but 1 subject (3%) had beta2-AAB. Chagas’ patients with cardiomyopathy were positive to 100% for beta1-AAB, to 89% for beta2-AAB, and to 98% for M2-AAB. In contrast, Chagas’ patients with megacolon were positive to 97% for beta2-AAB and to 100% for M2-AAB, but only to 38% for beta1-AAB, which is significantly lower than in the cardiomyopathy patients. Patients with both diseases possessed all 3 AABs to nearly 100%. In consequence, Chagas’ cardiomyopathy or megacolon patients are always detected by 1 or both of the autoantibody combinations beta1-/M2-AAB and beta2-/M2-AAB.

In the indeterminate state, 34%, 33%, and 42% of the patients were positive for beta1-, beta2-, and M2-AAB, respectively. With respect to the AAB compositions, 34% of the asymptomatic patients were positive either for beta1-/ or beta2-/M2-AAB or even for both AAB combinations.
or moderate cardiomyopathy to patients with severe cardiomyopathy (Fig. 3).

Discussion

We found that nearly all chronic Chagas' patients with cardiomyopathy and/or megacolon had AAB against G-protein–coupled receptors such as beta1-, beta2-, and M2-AAB, which agrees with recently published findings (10–12). Additionally, we showed specifically composed patterns of beta1-, beta2-, and M2-AAB in chronic Chagas' patients.

An AAB pattern frequently composed of beta1-, beta2-, and M2-AAB was indicative for Chagas' cardiomyopathy. Megacolon patients had beta2- and M2-AAB, but with lower frequency beta1-AAB. Aside from the distinguished composition of the AAB pattern in Chagas' cardiomyopathy and megacolon, we also showed different levels of the AAB that were higher for beta1- and M2-AAB in cardiomyopathy and for beta2-AAB in megacolon. Based on receiver-operator characteristic analysis, a cutoff of 8.9 U for beta1-AAB is the best way to distinguish between patients with cardiomyopathy and those with megacolon. We then showed that nearly one-third of the asymptomatic patients, thus those being in the indeterminate state, already carried AAB. Interestingly, the frequency of AAB positivity in the indeterminate state parallels the epidemiological situation that nearly 30% of infected subjects develop clinical manifestations (4–7).

Additionally, the frequency of 90% of the AAB-positive patients in the indeterminate state who showed a cardiomyopathy-typical beta1-AAB level, whereas 10% showed the megacolon level, again mirrors the epidemiological data for patients moving from the indeterminate state to Chagas' cardiomyopathy and megacolon, respectively (4–7). All of these findings for AAB in chronic Chagas' patients manifest in our view the hypothesis of AAB as a pathogenic driver for clinical manifestations such as cardiomyopathy and megacolon in Chagas' disease. The first-time demonstration of relationships between the beta1-AAB and partially of the M2-AAB level and the severity of cardiomyopathy also supports the pathogenic function of AAB against G-protein–coupled receptors in chronic Chagas' disease, which has already been deduced from experiments showing potentially pathogenic interactions of AAB isolated from Chagas' patients with cells and specimens obtained from animals and humans (8,13–16). Most impressive in this context is, due to the cross-talk between beta1-AAB of Chagas' patients and patients with idiopathic dilated cardiomyopathy (17), the induction of cardiomyopathy in rats after the transfer of idiopathic dilated cardiomyopathy beta1-AAB (18).

Furthermore, the concordance between the epidemiological data for heart and gastrointestinal disease in Chagas' patients and the frequency of asymptomatic patients carrying typical cardiomyopathy or megacolon AAB patterns for the first time offer an indication that measurement of AAB in asymptomatic Chagas' patients could be helpful in finding those indeterminate patients who have a high risk of developing the life-threatening complication of Chagas' disease. This would allow earlier patient enrollment in care programs to prevent or delay the clinical manifestations in Chagas' disease. However, our suggestion of using AAB against G-protein–coupled receptors as risk markers in Chagas' patients is preliminary and needs prospective studies for substantiation.

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REFERENCES


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