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Combined analysis of cross-reacting antibodies anti-β1AR and anti-B13 in advanced stages of Chagas heart disease

Luz M. Rodeles[1,2,a*], Miguel H. Vicco[1,a], Iván A. Bontempi[1], Alvaro Siano[3], Georgina Tonarelli[3], Oscar A. Bottasso[5], Pablo Arias[4], Iván S. Marcipar[1]

[1] *Laboratory of Immunological Techniques, National University of Littoral, Santa Fe, Argentina*
[2]*Internal Medicine Department, National University of Littoral, Santa Fe, Argentina*
[3] *Organic Chemistry Departament, National University of Littoral, Santa Fe, Argentina*
[4]*Human Physiology Department, National University of Rosario, Rosario, Argentina.*
[5]*Institute of Clinical and Experimental Immunology, UNR-CONICET, Rosario, Argentina*

[a] Both authors contributed equally to this study.

Abstract

Objective: Autoantibodies cross-reacting with the β1 adrenergic receptor (anti-β1AR and anti-p2β) and cardiac myosin antigens (anti-B13), have been related to the pathogenesis of chronic Chagas heart disease (CCHD). Studies exploring their levels in different stages are scarce. We aimed to evaluate the relationship of these autoantibodies with the clinical profile of chronic patients, especially regarding their classificatory accuracy in severe presentation with heart failure.

Methods and results: We conducted a cross-sectional study of 155 *T. cruzi*-seropositive patients and 26 age- and gender-matched healthy controls. They were categorized in three stages of CCHD. Serum antibodies were measured by specific immunoassays. Symptomatic individuals showed increased levels of anti-β1AR and anti-B13, while anti-p2β antibodies were similar between groups. A composite logistic regression model including anti-B13, anti-β1AR antibody levels and age, was able to predict systolic heart failure yielding an area under the curve of 83% (sensitivity of 67% and specificity of 89%).

Conclusions: In our study, anti-β1AR and anti-B13 antibodies were higher in individuals with chronic Chagas heart disease stage III, mainly in those with dilated cardiomyopathy associated with systolic heart failure. Logistic regression analysis showed that both antibodies were good predictors of severe CCHD. As well as being involved in disease progression, anti-β1AR and anti-B13 antibodies may be used as a serum marker of poor prognosis in terms of heart compromise.

Keywords: beta-1 Adrenergic Receptors, anti-B13 antibodies, Chagas disease, heart failure.

Introduction

Chagas disease, caused by the protozoan *Trypanosoma cruzi,* remains one of the most important causes of cardiac related mortality and disability in endemic areas. According to the latest WHO report, 6 million people are affected in Latin America. However, due to immigration processes, the disease is extending its distribution towards non-endemic areas such as the US and some European countries[1]. Chronic Chagas heart disease (CCHD) constitutes its most frequent and serious presentation, developing in approximately 30% of infected non-treated individuals. In advanced stages, it comprises different kinds of manifestations including dilated cardiomyopathy with heart failure (HF), arrhythmias, heart blocks and even sudden death[2].

Although it has been widely disclosed that inflammatory response plays a major role in CCHD, humoral autoimmune response seems to be related to clinical progression of these patients. Several authors have identified molecular mimicry between some parasitic and host's proteins. This mechanism originates some cross-reacting IgG as anti-B13, anti-p2β and anti-β1AR (β1 adrenergic receptor) antibodies that have been explored both in humans and in experimental models[3-5].

It has been demonstrated that *T. cruzi* B13 antigen promotes T cell cross-reactive responses due to sequence homology of part of this protein with a hexapeptide belonging to human cardiac myosin heavy chain[3]. Furthermore, these antibodies were present in patients with severe CCHD but rarely detectable in asymptomatic ones[6].

As regards anti-p2β antibody, this cross-reacts against the second extracellular loop of the β1 adrenergic receptor that bears similarity to specific antigenic epitopes on parasitic ribosomal proteins p2β and P0[7-10]. Experimental studies have shown that these autoantibodies exert an agonistic effect on the β1AR inducing myocyte apoptosis, increased heart chronotropism, and electrocardiographic alterations[8, 11-13]. Furthermore, Diez et al. have suggested that the presence of severe CCHD coexisted with higher levels of these antibodies[14].

Finally, anti-β1AR antibodies were observed in individuals with dilated cardiomyopathy. Similarly as anti-p2β, anti-β1AR also have shown in experimental models that cross-react with β1AR inducing the same effects[8, 9]. However, an association between anti-β1AR antibodies and the clinical presentation of Chagas disease has never been explored.

In view of the long asymptomatic period that characterizes chronic *T. cruzi* infection and the pathogenic role autoantibodies, we hypothesize that they might be suitable biomarkers for early recognition of chronic indeterminate subjects with higher risk of CCHD progression. In these patients, reinforcement of preventive actions (or even specific parasitic treatment) could be particularly considered.

Therefore, we conducted the present study to ascertain the relationship of anti-β1AR and anti-p2β antibodies with the clinical profile of CCHD patients. We also explored the interactions between these antibodies with previously studied anti-B13 in order to characterize their integrated classification performance for HF, as a first approach to their potential prognostic utility.

Materials and methods

Study population and subject evaluation

In this cross-sectional observational study, 155 *T. cruzi*-seropositive patients from Santa Fe, Argentina, were prospectively included. Most were born in rural endemic areas in the north of the country. Another 26 age and gender matched healthy subjects were considered for the control group. Participants were recruited from 2012 to 2015 at the Internal Medicine Department of the J. B. Iturraspe Hospital. Complete clinical evaluation included physical examination, 12-lead ECG recording, chest and abdominal X-rays and biochemical tests. Doppler echocardiography was also performed according to Acquatella et al to characterize CCHD patients[15]. On the basis of a well-established classification proposed by Storino et al[16], they were grouped as follows: (a) clinically asymptomatic subjects with normal radiological, ECG and echocardiography studies (CCHD I; n= 59); (b) patients with ECG alterations (i.e., left anterior fascicular block, left or right bundle branch block, total atrioventricular block, complex ventricular arrhythmias) and/or echocardiographic impairment without HF (CCHD II; n= 54); and (c) patients with clinically manifested heart failure and/or dilated cardiomyopathy by transthoracic echocardiography (CCHD III; n=42). Stages II and III are considered symptomatic CCHD as patients present evidence of cardiac disorders. The diagnosis of clinic and echocardiographic HF was defined made to the European Society of Cardiology guidelines for the diagnosis and treatment of acute and chronic HF 2012[17]. Dilated cardiomyopathy was considered when left ventricular en-

largement was associated with <45% ejection fraction and/or <25% of fractional shortening. Regarding HF treatment, it was specially assessed whether the patient had been taking β1-selective blockers at least for the 6 previous months at doses ranging from 5 to 10 mg/day for bisoprolol or 50 to 100 mg/day for atenolol.

The following exclusion criteria were considered: a) previously known coronary artery disease, or coronary artery disease equivalents such as diabetes mellitus or peripheral arterial disease; b) history of other cardiac diseases; c) systemic complaints; and d) previous or present treatment with anti-*T. cruzi* compounds or immunosuppressive drugs.

Written consent was obtained from all patients after they had been fully informed about the characteristics of the study. This research protocol was approved by the Ethics Review Board of J. B. Iturraspe Hospital and National University of the Littoral. The procedures followed the ethical standards given in 1964 Declaration of Helsinki and its later amendments.

Proteins production and purification

For obtaining proteins used as coating antigens, *Escherichia coli* BL21(DE3) cells bearing the plasmidic constructions pET-32a/B13 or pET-32a/p2b, respectively, were grown overnight in LB medium, supplemented with 0.1 mg/ml ampicillin at 37°C with agitation. Protein expression was induced in 1 mM isopropyl-β-D-thiogalactopyranoside and was purified with a Ni-nitrilotriacetic acid Sepharose column (GE Healthcare, UK) [18, 19].

The H26R peptide (H-W-W-R-A-E-S-D-E-A-R-R-C-Y-N-D-P-K-C-C-D-F-V-T-N-R-C), corresponding to the second extracellular loop of the human β1AR, was synthesized as a C-terminal amide by Fmoc solid-phase. Couplings were performed by N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]- N-methylmethanaminiumhexafluorophosphate N-oxide (HBTU) and diisopropylethylamine (DIEA)[20]. Fmoc-removal was done with 20% piperidine in DMF (v/v). Final cleavage from the resin was achieved by a mixture of TFA/H_2O/1,2-ethanedithiol (EDT)/triisopropylsilane (TIS) (94.5: 2.5: 2.5: 0.5) (v/v). After 4.5 h, the resin was filtered off and the crude peptide was precipitated in dry cold diethyl ether, centrifuged, and washed with cold diethyl ether until the scavengers were removed. Then the product was dissolved in H_2O and lyophilized twice. The synthetic analogs were analyzed and purified by reverse phase HPLC (Waters, USA) and Maldi-TOF.

Assessment of autoantibodies

Serum samples from patients were stored at -80°C until they were used for the measurement of antibodies against p2β, B13 and β1AR by specific immunoassays (ELISA) as previously described technique[18-20]. For assay standardization, results were expressed as an Index of Optical Density (IOD) of the sample in relation to the negative control (mean+2DS); an IOD ≤1 was considered negative.

Statistical analysis

Data were analyzed with MedCalc 12.2.1 (MedCalc Software, Belgium) and Graph Pad 5.03 (GraphPad software, USA). Normally distributed continuous variables are expressed as means ± SD. Chi-square or Fisher's exact tests were used for comparing categorical variables, whereas Student's non-paired T test or a one-way ANOVA (Student-Newman-Keuls test for all pairwise comparisons), as considered appropriate, were used to compare means. Non-parametric tests were employed for comparison provided continuous variables not normally distributed. Pearson's correlation coefficient and adjusted R^2 were determined for estimating the association between two quantitative variables.

The association of the variables with the presence of symptoms and diagnosis of systolic HF was assessed. A multiple binary logistic regression model step-forward was performed combining variables that where both univariate and multivariate correlated to HF. Then we applied the bootstrap method in order to make an internal validation of the model in half of the sampled individuals. In addition, we plotted the discriminating capacity of the antibody levels to predict this endpoint in a Receiver Operating Characteristic (ROC) curve. In all cases, a p value < 0.05 was considered significant.

Results

Clinical profile and antibody status

Clinical features and humoral immune response to the studied antigens of the sampled CCHD individuals and controls are summarized in Table 1. Symptomatic patients (CCHD stage II and III) presented increased levels of anti-β1AR and anti-B13. Positivity for anti-β1AR (IOD >1) was also associated with symptomatic CCHD stages (*p*<0.0001). On the other hand, as depicted in the table, anti-p2β IOD did not differ significantly among these categories. All control patients were negative for the above mentioned antibodies.

Patients of CCHD stage III were older than the asymptomatic ones. To assess the potential relevance of this issue in relation to antibody levels, we performed a simple linear regression between age and anti-β1AR IOD obtaining a very poor association (R^2 0.031; p=0.029). Regarding anti-B13, the correlation was not significant.

Hypertensive CCHD individuals (n=63) showed higher anti-p2β antibodies (6.78±4.40 vs 4.82±3.34; p=0.004) while anti-β1AR levels were similar to the non-hypertensive ones. Otherwise, hypertension and the presence of systolic HF, were associated (p<0.001).

Related to HF, 24 out of 42 patients with this condition, were receiving β blockers (β1 selective blockers=18; unselective β-blocker=6). However, anti-β1AR and anti-B13 IOD were similar compared to patients untreated with these drugs (1.37±0.523 vs 1.43±0.401; p=0.673 and 7.03±2.87 vs 6.28±3.59; p=0.455, respectively). Conversely, anti-p2β levels were higher in individuals treated with β blockers (p<0.05). Analysis upon excluding patients under β blockers treatment showed no relation between CCHD stage and anti-p2β.

ECG and ecocardiography findings

The most common alterations on ECG were left anterior fascicular block with right bundle branch block (n=32), atrial fibrillation (n=18), right bundle branch block (n=11) and right bundle branch block with atrial fibrillation (n=9). While anti-p2β IOD did not show variations, both left anterior fascicular block with right bundle branch block and right bundle branch block with atrial fibrillation, were associated with higher IOD of anti-β1AR and anti-B13 (p=0.02).

Antibody levels according to different echocardiographic findings are displayed in Table 2. Individuals with dilated CCHD and reduced ejection fraction showed increased anti-B13 and anti-β1AR antibodies as represented in Figure 1. Again, anti-p2β levels remained similar between CCHD groups.

Diagnostic performance in patients with heart failure

We applied a binary logistic regression model step-forward to predict dilated cardiomyopathy associated with systolic heart failure employing the variables previously related to this outcome i.e., age, IOD of anti-B13, IOD of anti-β1, and hypertension. As stated above, levels of anti-P2β were excluded, as were other potential confounding variables (i.e., smoking, sex) due to an insignificant bivariate association. The analysis revealed that anti-β1, anti-B13 and age contributed significantly to the prediction of this outcome (Table 3; Hosmer &Lemeshow test of 4.8; p=0.778). Internal validation by bootstrap sample (n=78) yielded similar results.

A ROC curve of anti-β1AR for systolic HF was obtained with an area under the curve (AUC) of 71% (95% IC 63.02 - 78.31, p<0.001). At a cut off value of 1.19, sensitivity was 76.67% (95% CI: 57.7 – 90.1) and specificity 68% (95% CI: 59.1 – 76.1). Concerning anti-B13, its AUC was 69% (95% IC 62 - 77, p=0.0002) for a 4.32 associated criterion, with sensitivity of 83.33% (95% CI: 65.3 – 94.4) and specificity of 50% (95% CI: 40.9 – 59.1). There was no difference between both ROC curves.

The proposed logistic regression model for systolic HF showed an AUC of 83% (95% IC 77 - 89, p<0.001) with a sensitivity of 67% and specificity of 89% (Table 3). Comparison between the ROC curves revealed that the AUC of the logistic regression model was significantly higher than the corresponding individual antibodies (Figure 2).

Discussion

Cellular and humoral immune responses constitute intermingled pathological mechanisms dealing with the visceral compromise in *T. cruzi* infection[21-22]. It has been argued that autoimmune response, due to bystander or molecular mimicry between host and parasite proteins, plays a pathogenic role in the development of CCHD[3, 23]. In this regard, as a first approach, our study aimed to ascertain the levels of antibodies induced by *T. cruzi* regarding pathological clinical manifestations and their potential diagnostic performance in discriminating asymptomatic patients from those with severe forms of the disease.

Regarding anti-p2β, Diez et al suggested that the presence of severe CCHD coexisted with higher levels of these antibodies[14]. Conversely, in our study with a larger sample of individuals per group, this association was not found since anti-p2β antibodies did not differ significantly among the CCHD stages. We have previously described that intake of cardio selective β-blocker drugs to improve survival in systolic heart failure, as seen in individuals with Chagas disease, was related to higher levels of anti-p2β[19]. Possibly the increased anti-p2β levels of individuals with CCHD stage III reported by Diez et al. might have to do with this pharmacological treatment as these data was not analyzed in this paper. Beyond this issue, our study fails to support a pathological effect of anti-p2β antibodies on heart tissue. Novel prospective studies should be performed to clarify the controversy about the effects and utility of anti-p2β in Chagas disease.

Concerning anti-B13 antibodies, Tibbetts et al reported that infection by *T. cruzi* may induce a myosin antigen-specific autoantibody associated with chronic inflammatory cardiomyopathy[24]. As well as promoting T cell cross-reactive responses against human cardiac myosin heavy chain, anti-

B13 is a well-documented serological test, which might be used as an earlier sera marker of response to anti *T. cruzi* treatment in chronic Chagas disease[25]. Cunha-Neto et al observed the presence of cardiac myosin-B13 cross-reactive antibodies in Chagas patients with heart failure[6]. Confirming and extending our observations[26], increased anti-B13 titers are also seen in patients with echocardiographic diagnosis of systolic heart failure. Analysis by means of the logistic regression model confirms that anti-B13 can be regarded as a predictor of chronic Chagas heart disease.

Anti-β1AR was first described several years ago in an experimental model of Chagas disease[5]. Ferrari et al have proposed that they stem from the molecular mimicry between the immunodominant protein P0 of *T. cruzi* ribosome[27]. Several studies have proven their agonist effect on the β1AR inducing heart rhythm disturbances and left ventricular dysfunction in murine models[9, 10, 28]. However, in the work performed by Talvani et al, in which all *T. cruzi*-infected individuals had anti-β1AR antibodies no association between their levels and clinical manifestations was found[29]. Conversely, the study by Wallukat et al, recruiting a larger sample of individuals serologically positive for *T. cruzi*, showed that most asymptomatic individuals were anti-β1AR negative; whereas all cases with Chagas cardiomyopathy had positive levels of anti-β1AR[30]. However, they did not explore whether anti-β1AR levels showed a relationship with the degree of myocardial compromise.

In this regard, the main contribution of our work is to provide a more extensive data on the relationship between increased anti-β1AR and anti-B13 antibodies with CCHD. As commented, both autoantibodies may be helpful in early identification of asymptomatic individuals at risk of developing severe CCHD. In addition, it would be interesting to include them in more complex models employing other independent inflammation and cardiac-damage markers reported recently (i.e., *T. cruzi* parasitemia by *RT*-PCR, NT-proBNP)[31].

Noteworthy is the fact that given the slow clinical progression of Chagas disease, even over decades, it was necessary to begin with a cross-sectional design as an initial attempt to assess the implications of these possible CCHD progression markers. We tried to achieve a significant sample size for a better study power, constituting a central contribution of the present work in relation to other studies in the literature.

Present studies should be expanded to other endemic scenarios to ultimately validate the usefulness of anti-β1AR and anti-B13 antibodies as an additional tool allowing a better control of at-risk patients, especially younger ones.

This was a single-center study and due to its design, we cannot extend our results to what happens in the evolution of the disease regarding antibody titers as patients change their CCHD stage. A longitudinal follow-up approach is necessary to confirm our findings.

Conclusions

In summary, our results suggest a significant association of anti-β1AR and anti-B13 antibodies with severe symptomatic Chagas heart disease. In addition, both estimations showed to be potential heart failure predictors by the regression model. Also, anti-β1AR and anti-B13 antibodies seem suitable for further validation in an prospective cohort.

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Corresponding author: Luz M. Rodeles, Laboratory of Immunological Techniques, Faculty of Bio-chemistry and Biological Sceinces, National University of Littoral, Ciudad Universitaria, CC242, Santa Fe, CP 3000, Argentina. Phone +54-342-4575215, Email luzrodeles@gmail.com

Table 1: Characteristics of chronic Chagas heart disease patients by group

Features	Control (n=26)	CCHD I (n=59)	CCHD II (n=54)	CCHD III (n=42)
Age	50.1±13.54	45.4±13.13[a]	53.5±11.04	58.8±9.78[**a**]
Male/Female (n)	12/14	19/40[b]	23/31[b]	25/17[**b**]
BMI (kg/m^2)	26.8±2.94	27.9±5.73	26.9±2.61	25.9±3.03
Smoking % (n)	25% (5)	17% (10)	24% (13)	31% (13)
Heart rate (bpm)	76.7±10.93	75.4±9.00	68.6±7.62	70.6±15.12
Hypertension % (n)	27% (7)	20% (18)[**c**]	40% (36)[c]	40% (36)[c]
SBP (mmHg)	130 (130-141)	120 (110-132)[**d**]	130 (124-132)	142 (130-144)[d]
DBP (mmHg)	84 (80-90)	78 (70-82)	80 (80-90)	80 (78-90)
Antibodies (IODN)				
Anti-B13	0.645±0.237[e]	4.40±2.67[e,**f,g**]	4.928±2.75[e,**f,g**]	6.71±3.18[e,**f,g**]
Anti-β1AR	0.608±0.208[h]	0.887±0.501[l,j]	1.12±0.507[h,j]	1.41±0.470[i,h,**j**]
IODN anti-p2β	0.802±0.210[k]	5.64±4.09[k]	5.84±4.03[k]	5.20±3.6[k]

Data are expressed as percent (number), mean ± SD or median (interquartile range), as appropriate. CCHD: Chronic Chagas Heart Disease. SBP: Systolic blood pressure. DBP: Diastolic blood pressure. Letters indicate main significant differences (underlined) regarding the variables marked with the same character. a, c, d, e, h: $p<0,05$; b, f, g, I, j: $p<0,01$.

Table 2: Mean values of auto antibodies levels according echocardiographic features

Categories	Anti-B13	Anti-β1AR	Anti-p2β
Dilated CCHD with reduced EF (n=30)	7,06±3,33[a,b]	1,41±0,46[c,d]	4,97±3,06
AS hypokinesis/aneurism with HF (n=12)	5,84±2,67	1,34±0,49	5,78±4,84
AS hypokinesis/aneurism (n=4)	5,75±2,85	0,91±0,54	6,99±4,03
Concentric ventricular hypertrophy (n=8)	3,53±2,38[b]	0,88±0,39[d]	2,68±0,81
Normal echocardiogram (n=98)	4,74±2,73[a]	1,01±0,53[c]	4,11±0,41

CCHD: Chronic Chagas Heart Disease. EF: ejection fraction. HF: Heart failure. AS: apical septal. Letters indicate main significant differences (underlined) regarding the variables marked with the same character. **a, c**: $p<0,002$; **b:** $p=0,005$; **d**: $p=0,037$.

Table 3: Logistic regression model to predict systolic HF

Variables included	OR (95% CI)	*Sig*.
Anti-β1AR	3,22 (1.35-7.69)	*p=0.008*
Anti-B13	1.27 (1.10-1.47)	*p=0.001*
Age	1.06 (1.02-1.11)	*p=0.002*
Sensitivity 67% (47.2 – 82.7)		
Specificity 89% (82.9 – 94.3)		

Figure Legends

Figure 1: Anti-B13 (A) and anti-β1AR (B) autoantibody levels in patients according to the presence of heart failure diagnosed by echocardiography.

CCHD: Chronic Chagas Heart Disease; HF: heart failure; IOD: index of optical density. *$p<0.002$; **$p<0.001$

Figure 2: Receiver Operating Characteristic curve analysis of anti-β1AR, anti-B13 auto antibodies and their combined use in the regression model.

AUC of the logistic regression model was significantly higher ($p<0.001$).

A

B