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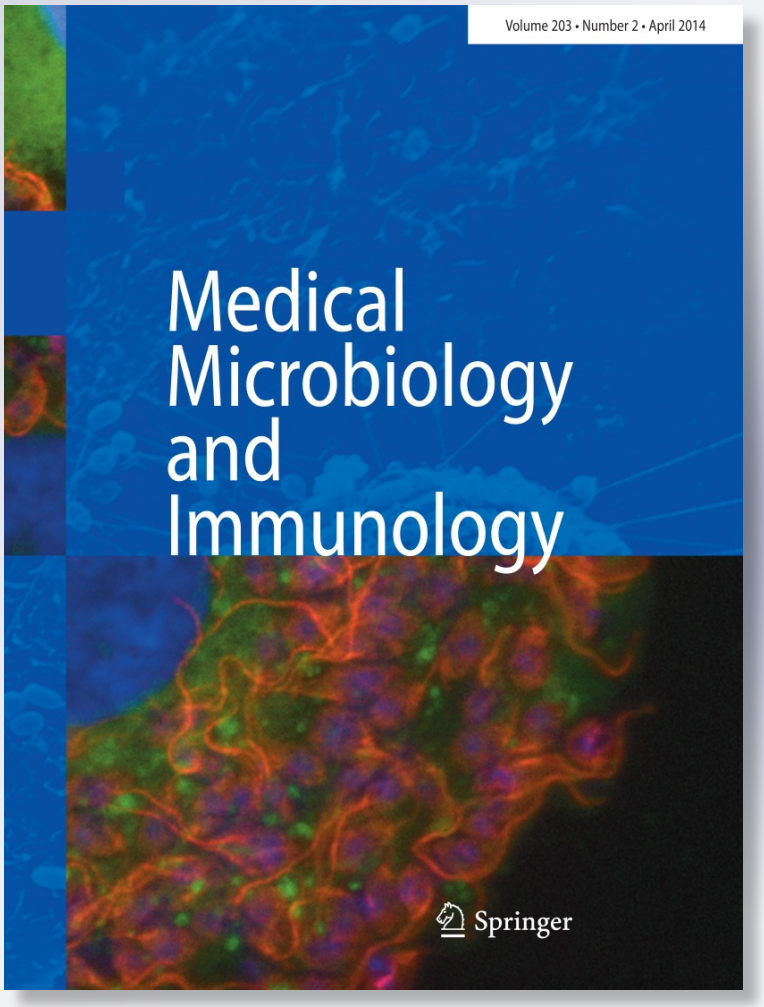

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ORIGINAL INVESTIGATION

Decreased level of antibodies and cardiac involvement in patients with chronic Chagas heart disease vaccinated with BCG

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Abstract Studies indicate that *Trypanosoma cruzi* is capable of inducing immunological disturbances such as decreased expression of molecules involved in T-cell survival and costimulation for antigen-driven T-cell responses. On the other hand, several reports have described that BCG vaccination induces a T-helper 1-type immune response with protective effects in different pathologies. In this regard, we evaluated whether BCG vaccination coexists with a better clinical and immunological profile of chronic Chagas heart disease (CCHD). We performed a cross-sectional study in *T. cruzi* seropositive patients categorized according the BCG vaccine background and to the well-established CCHD classification provided by Storino et al. All individuals were subjected to a complete clinical examination. All patients presented detectable levels of autoantibodies anti-p2β, anti-B13, anti-FRA and antiparasite homogenate immunoglobulins, which were unrelated to age and sex distribution or blood pressure values. Comparisons according to BCG vaccination revealed that individuals who had not been vaccinated presented higher values of antibodies, and patients without BCG vaccine had an OR of 6.1 (95 % CI 1.23–29.25, $p = 0.02$) for globally dilated cardiomyopathy with reduced ejection fraction (Hosmer and Lemeshow test of 5.2 $p = 0.73$). Our results suggest that BCG vaccination coexists with a better clinical and immunological profile of CCHD, associated with lower cardiac involvement.

Keywords Chagas disease · *T. cruzi* · Heart failure · Autoantibodies · BCG vaccine

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Introduction

Chagas disease is a parasite infection caused by the protozoan *Trypanosoma cruzi* that affects at least 8–10 million people worldwide. Although mega syndromes involving the gastrointestinal tract can be detected in chronically infected patients, chronic Chagas heart disease (CCHD) continues to be the most common manifestation, affecting about 30 % of chronically infected individuals and causing approximately 50,000 deaths annually [1, 2].

Nowadays, it is considered that the immune response to parasite antigens, the autoimmune processes, and the inflammatory response are interrelated pathological mechanisms involved in the development of CCHD [3–10]. *T. cruzi* is known to promote humoral autoimmune responses due to the molecular mimicry displayed by some parasite proteins. Among the several autoantibodies that have been described, anti-p2β and anti-B13 have been shown to play a pathogenetic role in the development of heart tissue lesions, both in humans and in animal models [10–12]. Anti-p2β antibodies cross-reacting against the β1 adrenergic receptor (β1-AR) have been involved in an increased chronotropic effect and myocyte apoptosis [13–16]. In turn, anti-B13 antibodies were shown to promote a cellular response to cardiac myosin because of the sequence homology to B13 protein epitope of human

cardiac myosin heavy chain hexapeptide [10, 17, 18]. On the other hand, *T. cruzi* is capable of inducing immunological disturbances such as decreased expression of molecules involved in T-cell survival and costimulation for antigen-driven T-cell responses [19, 20], and inhibition of interleukin two receptors expressions [21], among others. These immunological disturbances are associated with the survival of protozoa in the host and the persistence of inflammatory response.

Within the setting of defensive mechanisms against intracellular pathogens, several reports have described that BCG vaccination induces a T-helper 1-type immune response with protective effects in different pathologies such as tuberculosis, leprosy and some types of cancer [22–26]. In relation to Chagas disease, Bertelli et al. [27] reported that BCG vaccination induces resistance to an infective challenge with *T. cruzi* in a murine model. No studies have yet ascertained whether BCG vaccination coexists with a better clinical and immunological profile of CCHD. To ascertain this question, the present study was carried out.

Materials and methods

We performed a cross-sectional study in 110 patients with proven diagnosis of Chagas disease [28], recruited at the Clinical Service of the Iturraspe Hospital, Santa Fe, Argentina. All of them were subjected to a complete clinical assessment. The patient was considered to be BCG vaccinated, if the BCG vaccine scar was present, as recommended in former studies [29]. Exclusion criteria included: (a) presence of two or more risk factors for coronary artery disease (e.g., family history of early heart disease, hypertension, high blood cholesterol levels, obesity and metabolic syndrome). Patients with diabetes or peripheral arterial disease were also excluded considering that both diseases are coronary artery disease equivalents; (b) history of established coronary artery disease; (c) history of other cardiac diseases, e.g., *cor pulmonale*, valvular heart disease, rheumatic heart disease, toxic cardiomyopathy and congenital heart disease; and (d) any other systemic complaints. Individuals treated with anti-*T. cruzi* compounds or immunosuppressive drugs were excluded, as well. The study was approved by the Ethics Review Board of Universidad Nacional del Litoral. Informed consent was obtained from all participants.

On the basis of a well-established CCHD classification [30], individuals were classified as follows (a) 30 individuals with CCHD stage I, i.e., normal radiological, ECG and echocardiography studies, (b) 40 patients with CCHD stage II, i.e., with ECG alterations but without heart failure and (c) 40 patients with CCHD stage III, namely clinical heart failure and/or dilated cardiomyopathy by transthoracic echocardiography (21 individuals from this group had a recent diagnosis of CCHD). The echocardiogram procedures were performed according to Acquatella et al. [31], and M-mode, two-dimensional echocardiography and Doppler ultrasonography were used.

Dilated cardiomyopathy criteria were the presence of LV ejection fraction <45 % and/or fractional shortening <25 %, associated with LV enlargement, defined as LV end-diastolic diameter ≥117 % of the predictive value corrected for age and body surface. Clinical heart failure was defined according to the guidelines for the diagnosis and treatment for acute and chronic heart failure of the European Society of Cardiology [32]. The risk score for predicting death in Chagas heart disease [33, 34] was also employed.

Protein expression, purification and measurement of antibodies

Total homogenates of epimastigotes (TH) were obtained by resuspension of the washed cells in five volumes of 1 mM Nα-p-tosyl-L-lysine chloromethyl ketone and 1 mM phenylmethylsulfonyl fluoride (PMSF) in distilled water. For p2β and FRA proteins production, *E. coli* BL21(DE3) cells bearing the plasmidic constructions pET-32a/p2β and FRA were grown overnight in LB medium, supplemented with 0.1 mg ml/1 ampicillin at 37 °C, with agitation. Protein expression was induced in 1 mM isopropyl-β-D-thiogalactopyranoside and purified with a Ni-nitrilotriacetic acid column (GE), as described elsewhere [35].

Serum samples were obtained by conventional venipuncture, and antibodies were measured by immunoassay (ELISA). Microtiter plates were coated with 0.5 μg of specific antigens (p2β, FRA and homogenate parasites) in 0.05 M carbonate–bicarbonate buffer, pH 9.6 and incubated overnight at 4 °C. Plates were washed thrice with 0.01 % Tween in PBS and were blocked with 5 % bovine serum albumin and incubated with a 1:100 dilution of human serum. Microplates thus sensitized were incubated with a 1:100 dilution of human serum in 1 % skimmed milk in PBS at 37 °C for 60 min. The plates were washed, and peroxidase-conjugated goat antihuman IgG (Sigma, St. Louis, MO) was added. Plates were read at 450 nm in an ELISA reader (Maxiline Microplate Reader; Invitrogen, Carlsbad, CA) after incubation with trimethylbenzidine in H_2O_2. For each specific antigen, the results were expressed as the mean of the optical density (OD) of two simultaneous assessments of the same serum sample. In each plates and for each specific antigens, 6 negative controls (healthy individuals seronegatives for *T. cruzi*) were assayed simultaneously. ELISA negative standard cutoff values were calculated as the mean OD of the negative

serum samples plus two standard deviations. The levels of antibodies were expressed as the ratio between the OD of the sample and the OD of the negative standard cutoff. This index is referred as IODN (index of the optical density of autoantibodies in relation to the negative control). An IODN ≤ 1 was considered negative [36–38].

Statistical analysis

Data were analyzed with MedCalc version 12.2.1. Normal distributions of the continuous variables were tested by Kolmogorov–Smirnov method. The data are expressed as mean ± SD. Groups were compared in relation to age, antibody levels, CCHD stages and BCG vaccination condition. Chi-square test or Fisher's exact test were used for categorical variables, whereas the one-way ANOVA (Student–Newman–Keuls test for all pairwise comparisons) was used to compare means of IODN values and age among the groups defined by CCHD stage. A multiple binary logistic regression model was used to assess the impact of cardiovascular risk factors (hypertension, gender, smoking and alcohol consumption) associated with globally dilated cardiomyopathy and reduced ejection fraction. The Hosmer–Lemeshow test was employed for goodness of fit of the logistic regression model.

Results

Sampled individual characteristics

The sample was composed of 51 males and 59 females aged 55 ± 12.4 years (mean ± SD), with individuals from CCHD stage III being older than the remaining ones ($p < 0.05$). All of them were born in northern endemic rural areas and further migrated to Santa Fe city, where vectorial transmission is not present. There were no between-group differences as to sex distribution. Forty-six patients had essential hypertension not associated with other cardiovascular risk mentioned previously, all of them under drug-therapy (11, 17 and 17 cases from CCHD stage I, CCHD stage II and CCHD stage III, respectively). The proportion of individuals with hypertension among the CCHD stages was not statistically different, although systolic and diastolic blood pressure levels in CCHD I were significantly lower than values seen in the remaining groups [ANOVA F ratio 16.93 and 5.56, respectively, $p < 0.001$ (Student–Newman–Keuls test for all pairwise comparisons, $p < 0.05$)]. In relation to the transthoracic echocardiography, the pathological findings were left ventricular apical aneurysm in twelve patients. Two individuals from the CCHD II group had apical septal hypokinesis without diastolic or systolic disturbance, and two patients had concentric ventricular hypertrophy. Among the individuals with CCHD III, five of them had concentric ventricular hypertrophy with abnormal left ventricular relaxation left, five patients presented atrial enlargement and abnormal left ventricular relaxation with diastolic heart failure, and finally, 30 of them had globally dilated cardiomyopathy with reduced ejection fraction.

As regards BCG vaccination, there were 15 patients who had not been vaccinated. From these patients, 13 were categorized as CCHD III and 2 as CCHD II. In relation to vaccinated patients, 30, 38 and 27 cases belonged to CCHD stage I, II and III, respectively; the proportion of patient not vaccinated was higher in CCHD III compared with the other groups ($p < 0.001$). Further comparisons in relation to CCHD stage III and BCG vaccine background revealed that not vaccinated individuals had higher cardiac involvement by echocardiograph ($p = 0.03$). The characteristics of the patients in each group and the levels of antibodies are summarized in Tables 1 and 2.

Antibodies

All patients presented detectable levels of autoantibodies anti-p2β, anti-B13, anti-FRA and antiparasite homogenate immunoglobulins, which were unrelated to age and sex distribution or blood pressure values. No association between electrocardiographic conduction disturbances and IODN values for any of the tested autoantibodies was found. As shown previously [14], levels of anti-B13 antibodies were higher in CCHD stage III, in association with an increased heart involvement.

Mean ± SD values from the overall sample of vaccinated versus unvaccinated individuals for antiparasite homogenate, anti-FRA, anti-p2β and anti-B13 antibodies are shown in Table 3. Comparisons according to BCG vaccination revealed that individuals who had not been vaccinated presented higher values of antiparasite homogenate ($p = 0.0002$), anti-FRA ($p < 0.0001$), anti-p2β ($p = 0.0004$) and anti-B13 ($p < 0.0001$). Given our former demonstration that anti-p2β values were higher in patients under selective cardio β1 blockers [39], patients were then classified according β1 blockers intake and BCG vaccination background: (a) patients lacking both predictors, (b) patients with β1 blockers without BCG vaccine, (c) patients without β1 blockers but with BCG vaccine and (d) patients with both factors. The mean ± SD values for each group were 6.3 ± 2.6; 7.79 ± 2.17; 4.2 ± 2.32 and 5.1 ± 2.93, respectively. Individuals from the first two groups presented increased levels of anti-p2β, with patients under cardioselective β1 antagonist therapy but not BCG vaccine having the highest levels of anti-p2β, ANOVA F ratio 5.56, $p = 0.002$ (Student–Newman–Keuls test for all pairwise comparisons $p < 0.05$), Fig. 1.

Table 1 Characteristics of chronic Chagas heart disease patients by group

	Chronic Chagas heart disease			
	CCHD I ($n = 30$)	CCHD II ($n = 40$)	CCHD III ($n = 40$)	p
Age	47.8 ± 12	54.2 ± 11.6	61.5 ± 8.5	<0.001[a]
Gender (n)				
Male	12	19	20	NS
Female	18	21	20	
BCG vaccinated (n)	30	38	27	0.0003[b]
Systolic blood pressure	121 ± 9 mmHg	128 ± 10 mmHg	137 ± 16 mmHg	0.001[c]
Diastolic blood pressure	81 ± 11 mmHg	86 ± 8 mmHg	90 ± 9 mmHg	NS
Antibodies				
IODN-B13	4.178 ± 2.29	6.668 ± 2.167	9.95 ± 2.51	<0.001[d]
IODN-p2β	4.166 ± 2.935	5.071 ± 2.594	5.605 ± 2.553	NS
IODN-FRA	5.744 ± 3.523	5.274 ± 3.129	6.656 ± 2.969	NS
IODN-parasite homogenate	6.3511 ± 4.39	7.216 ± 4.29	6.656 ± 3.619	NS

Quantitative variables are expressed as mean ± SD

NS not significant difference; *IODN* index of the optical density of autoantibodies in relation to the negative control

[a] CCHD III different from CCHD I and CCHD II

[b] Proportion of unvaccinated patients of CCHD III is higher from CCHD II and CCHD I

[c] CCHD I different from CCHD II and CCHD III

[d] CCHD III different from CCHD I and CCHD II; CCHD II and III different from CCHD I

Table 2 Characteristics of chronic Chagas heart disease Stage III patients by BCG vaccination

	Chronic Chagas heart disease stage III		
	Not vaccinated ($n = 13$)	Vaccinated ($n = 27$)	p
Age	54.5 ± 3.53	60 ± 6.584	NS
Antibodies			
IODN-B13	7.793 ± 3.36	5.2 ± 1.55	<0.05[a]
IODN-p2β	5.605 ± 2.553	3.104 ± 2.377	
IODN-FRA	8.693 ± 2.797	3.667 ± 2.463	
IODN-parasite homogenate	7.025 ± 4.56	5.121 ± 1.626	
Echocardiographic pathological signs (n)[c]			
I	–	5	<0.05[b]
II	–	5	
III	13	17	

Quantitative variables are expressed as mean ± SD

NS not significant difference; *IODN* index of the optical density of autoantibodies in relation to the negative control

[a] Not BCG-vaccinated group presented higher antibody levels in all cases

[b] Patients not vaccinated presented higher proportion of globally dilated cardiomyopathy with reduced ejection fraction ($p = 0.032$)

[c] I: concentric ventricular hypertrophy with abnormal left ventricular relaxation left, II: left atrial enlargement and abnormal left ventricular relaxation with diastolic heart failure, III: globally dilated cardiomyopathy with reduced ejection fraction

Table 3 Levels of antiparasite homogenate, anti-FRA, anti-p2β and anti-B13 antibodies according to BCG vaccination from the overall sample of studied individuals (mean ± SD)

	Not BCG vaccinated ($n = 15$)	BCG vaccinated ($n = 95$)	p
IODN-B13	9.863 ± 3.56	5.216 ± 2.68	<0.0001
IODN-p2β	7.397 ± 2.165	4.213 ± 2.4	0.0004
IODN-FRA	10.176 ± 2.91	3.98 ± 2.861	<0.0001
IODN-parasite homogenate	11.1 ± 4.46	5.841 ± 2.60	0.0002

In a last attempt, a multiple binary logistic regression was conducted to predict globally dilated cardiomyopathy with reduced ejection fraction using the categorical variables BCG vaccine, hypertension, gender, smoking, alcohol consumption and the continuous variables age and IODN anti-B13. Calculations demonstrated that patients without BCG vaccine had an OR of 6.1 (95 % CI 1.23–29.25, $p = 0.02$) for globally dilated cardiomyopathy with reduced ejection fraction (Hosmer and Lemeshow test of 5.2 $p = 0.73$) Table 4.

Discussion

Contact with mycobacteria has long been recognized as an influential factor on the subsequent immune response

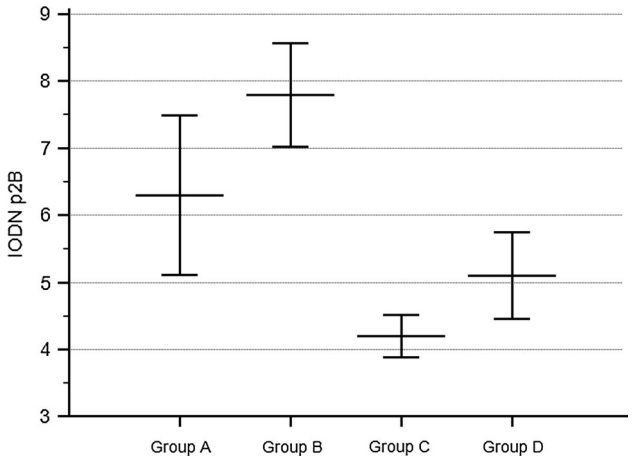

Fig. 1 Levels of IODN of anti-p2β according to the treatment group. *Lines* represent the means ± 1ES. Patients were classified according to β1 blockers intake and BCG vaccination background in: Group A patients lacking both predictors, Group B patients with β1 blockers without BCG vaccine, Group C patients without β1 blockers but with BCG vaccine and Group D patients with both factors. Patients of the Group B had the highest levels of anti-p2β, ANOVA F ratio 5.56, $p = 0.002$ (Student–Newman–Keuls test for all pairwise comparisons $p < 0.05$

Table 4 Summary of variables and odd ratios

Variables	Odd ratio (95 % CI)	*p* value
Age	1.04 (0.98–1.13)	0.16
Gender		
Male	1.14 (0.26–5.01)	0.8
Female	1.09 (0.17–2.33)	0.9
Smoking	1.14 (0.2–6.4)	0.8
Alcohol consumption	2.77 (0.55–13.8)	0.2
Hypertension	1.1 (0.19–6.64)	0.9
Autoantibodies anti-B13	2.1 (1.29–3.66)	0.001
Not BCG vaccinated	6.1 (1.23–29.25)	0.02

developed by the host [40, 41], partly because of the extraordinary adjuvant activity of aerobic Actinomycetales, of which the genus *Mycobacterium* is a member. As regards mycobacteria, they have unique and highly variable abilities to promote innate and adaptive responses including T-cell maturation, the classic Th1 and Th2 pathways, and the consequent production of different cytokine combinations. In the immunologically naïve individual, BCG regularly promotes Th1 maturation [42]. Such effects are likely to modify the response against related or unrelated antigens [43], which may account for the decreased mortality and morbidity seen in childhood and adulthood [44, 45].

Within the context that control of *T. cruzi* infection relies on the existence of a well-orchestrated defensive reaction, our studies indicate that chronically infected individuals presenting a BCG scar have a better course of their trypanosomiasis. Studies at the experimental level [27] showed that BCG vaccine induces a protective response against acute *T. cruzi* infection in mice. More recently, experiments in a well-established rat model of *T. cruzi* infection revealed that early immunization with heat-killed preparations of 2 related species of Actinomycetales, *Gordonia bronchialis* and *Rhodococcus coprophilus*, resulted in controlled parasitemia and ameliorated chronic myocarditis [46].

T. cruzi is likely to induce abnormal B-cell activation and imbalanced cellular immune response by decreased production of IL-2, essential for the expansion of antigen-specific clones of CD4 and CD8 T cells and NK cell proliferation [47–52]. In turn, BCG vaccination promotes persistent immune response mediated by increased levels of CD4 T cells, but also CD8 T cells and Treg [53, 54]. Interestingly, our current finding indicates that autoantibodies which may play a pathogenic role in Chagas disease are less present in BCG-vaccinated patients. Expanding our former demonstration that higher levels of anti-B13 were associated with increased heart damage [14], we now report that patients vaccinated with BCG presented decreased levels of anti-B13 and lower degree of heart damage. As regards anti-p2β antibodies, we found that the BCG vaccine status was also associated with lower concentration of anti-p2β. Decreased levels of antibodies also extended to the anti-*T cruzi* homogenate and anti-FRA (a specific constitutive antigen of *T. cruzi*) antibodies. Collectively, decreased autoantibody production may be the reflection of a favorable regulatory effect of BCG.

Considering the well-known effects of BCG on the immune response [22–26], vaccination with this mycobacterial species may be able by itself to promote a better disease outcome. Nevertheless, we cannot discard a mutually nonexcluding possibility of BCG vaccination being associated with a particular clinic and epidemiological situation in endemic areas. One of these potential differences may be *T. cruzi* linage infecting patients. As far as we know, except for TcI which is prevalent in northern Brazil, Central and North America, there are no gross differences as to the prevailing *T. cruzi* lineages and their clinical disease course in the Southern cone [55].

Studies in residents from endemic areas will help to corroborate the important association between BCG vaccine with decreased autoantibody levels and lower heart damage, also reinforced by the experimental evidence pointing out to a better disease course upon this kind of vaccination.

Limitation of the study

Our study was performed in a single center with a relatively reduced sample size. Also, because it is a cross-

sectional study, it is difficult to establish the true role of BCG vaccination in Chagas disease outcome.

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Conflict of interest These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation. There was no grant support or conflict of interests.

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