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Humoral Immune Response against P2β from *Trypanosoma cruzi* in Persons with Chronic Chagas Disease: Its Relationship with Treatment Against Parasites and Myocardial Damage

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Abstract. We investigated the relationship between potentially pathogenic antibodies against a *Trypanosoma cruzi* ribosomal protein (P2β) and the evolution of Chagas disease and the effect of trypanocidal treatment on these variables. Seventy-eight patients with chronic Chagas disease who were followed-up for more than 20 years were divided into three groups: 30 asymptomatic persons undergoing specific treatment (group A), 37 asymptomatic persons not undergoing specific treatment (group B), and 11 patients with chronic chagasic cardiomyopathy (CCC) who were not treated. Five patients in group B showed evolution to myocardial abnormalities. Among persons with CCC, six showed no changes; the remaining persons showed progression of cardiac involvement. Levels of antibodies to P2β in persons in group A decreased from their initial values. This finding was not observed in persons in groups B and C. Comparisons at the end of the follow-up showed lower amounts of antibodies to P2β in groups A and C. These findings support the benefits of specific treatment during chronic infection.

INTRODUCTION

Chagas disease or American trypanosomiasis is a protozoan infection caused by *Trypanosoma cruzi* and is endemic in Latin America. Recent estimates[1] indicate that this disease affects at least 8–10 million persons in South and Central America; there are sporadic cases in the United States and Canada. After infection, *T. cruzi* invades and multiplies within different host cells, including macrophages, smooth and striated muscles, fibroblasts, and neurons. In the absence of specific treatment, the symptoms of acute phase of Chagas disease persist for approximately two months, with a mortality rate of 2–8%, especially among children.

After resolution of acute infection, the indeterminate phase ensues and the patient shows strong evidence of immunity but remains infected. Some parasites evade the immune response and cause focal inflammatory lesions in several organs. Amastigote forms can be detected by conventional histologic and immunofluorescence and genomic markers can be detected by *in situ* hybridization.[2]

In the chronic phase that follows, most patients remain asymptomatic. However, the characteristic symptoms of this phase, cardiac, digestive, or neurologic disturbances, develop in approximately 20–50% of the patients, depending on the disease-endemic area.[3] The pathogenesis of chronic chagasic cardiomyopathy (CCC) is still controversial, but most researchers believe that the immune response contributes significantly to this pathology.

Different mechanisms have been proposed to explain the pathology of the cardiomyopathy that occurs in chronic Chagas disease. For example, parasite persistence not only results in chronic inflammatory reactivity, but also induces immune responses against parasite[4] and self-tissues[5] and the eventual damage accompanying these responses.[6–8] Several clinical reports reinforce the view of parasite persistence as being pathogenic.[9–12] The fact that signs of the disease are evident in tissues in which parasites are apparently absent support the autoreactive component. Cross-reactive antigens in heart muscle and *T. cruzi* have been demonstrated, but autoimmunity does not entirely explain Chagasic heart disease.

Several parasite structures and autoantigens seem to be involved in the pathology of Chagas disease. Among them, *T. cruzi* ribosomal proteins (P2β) are recognized by most serum samples from patients with chronic disease. Antibody levels against the P2β C-terminal region appeared to be related to the clinical status of chronically *T. cruzi*-infected patients.[13,14] Furthermore, antibodies against P2β are known to cause electrocardiographic alterations in non-infected hearts of immunized mice, which suggests possible involvement of these antibodies in experimental chagasic cardiomyopathy.[15,16] The immune response elicited by the parasite may be also modified by specific chemotherapy because interferon-γ-producing T cells specific for *T. cruzi* decreased after 12 months of treatment to become unnoticeable in many treated patients.[17]

Unlike cross-sectional studies in which predictors and outcome variables are measured on one occasion, longitudinal studies offer the opportunity of repeated measures to provide a better scenario for the study of relationships between personal characteristics or exposures and occurrence of health-related events. Within this setting, our center has detected and followed-up *T. cruzi*-seropositive patients; when recommended, some of these patients have also undergone specific treatment for Chagas disease. Information obtained has enabled us to ascertain whether the level of reactivity against P2β was associated with clinical evolution and to assess the effect of treatment against *T. cruzi* on the humoral response to P2β and some clinical correlates of disease outcome.

MATERIALS AND METHODS

Study population. This retrospective study was conducted with 78 patients who came to the Center for Research in National Endemic Diseases. The Center is located at the School of Biochemical Sciences, Littoral National University (Santa Fe, Argentina). All patients were born in rural areas of northern Argentina where Chagas disease is endemic and had migrated during their young adulthood to Santa Fe city. None of them had concomitant pathologic disorders

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(i.e., congenital or rheumatic cardiopathies and immunologic diseases) able to affect the end points being analyzed. Patients came to the center on a yearly basis and were evaluated by a clinical examination, specific serologic tests, frontal chest radiograph, and a 12-lead resting electrocardiogram (ECG), which was interpreted independently by an experienced cardiologist. Xenodiagnosis was also performed at the time of the initial visit and at regular intervals.

Presence of risk factors such as smoking, alcoholism, and hypertension was evaluated as in previous studies.[18] Smoking was defined as a 10-year history of at least 20 cigarettes per day. Alcoholism was defined as a daily intake of alcohol greater than 100 mg/day for a minimum period of 10 years. Hypertension was defined as a diastolic blood pressure ≥ 90 mm Hg.

Cardiac involvement was classified according to an established consensus (primarily the Kuschnir classification).[19] Group (G)0 was composed of asymptomatic persons with normal ECGs and chest radiographs. Group G1 was composed of persons with no congestive heart failure, but ECG showing any of following alterations: complete right bundle branch block and left anterior fascicular block in persons less than 50 years of age, right bundle branch block plus left anterior fascicular block, frequent ventricular extrasystole, ventricular extrasystole associated with conduction disorders, second-degree atrioventricular block, complete atrioventricular block, electrical inactivation areas (no antecedents of ischemic cardiopathy), and a chest radiographic cardiothoracic ratio < 0.50. Group G2 was composed of patients with pathologic ECG tracings as above, an abnormal chest radiographic cardiothoracic ratio > 0.50, and conserved functional capacity for light and moderate exercises. Group G3 was composed of patients with congestive heart failure, pathologic ECG tracings, and a chest radiographic cardiothoracic ratio > 0.50.

Patients analyzed in the present study corresponded to an initial sample population of 198 persons: 19 with cardiac complaints and 179 without symptoms; 63 of them received treatment for infection with *T. cruzi* (29 with nifurtimox [NIF] and 34 benznidazole [BZL]). Selected persons (n = 78, 53 females and 25 males, mean ± SD age = 31.5 ± 8.6 years) were those attending the center regularly (good compliers) who had well-preserved serum samples (see below).

Serum samples from 36 healthy controls (26 females and 10 males, mean ± SD age = 36.8 ± 9.2 years) and 16 patients with non chagasic cardiomyopathy (6 with ischemic origin and 10 with unknown etiology, 10 females and 6 males, mean ± SD age = 34.6 ± 7.8 years) were also analyzed for comparison purposes. Healthy controls and patients with non-chagasic cardiomyopathy underwent the same procedures, clinical evaluation, ECG, and radiographs.

Serologic analysis. Specific serologic analysis was performed by using enzyme-linked immunosorbent assay (ELISA), direct agglutination, indirect hemagglutination, and indirect immunofluorescence. Serum samples that showed positive reactions to all tests were considered positive. Serum samples from each control visit were treated with glycerol and stored at –20°C. Samples were regarded as suitable for the study purposes if the level of antibodies to *T. cruzi* from the test sample was similar to the one recorded in fresh serum samples obtained at the correspondent time point evaluation.

Treatment. Because a high frequency of parasites was an important guideline for therapy during the chronic phase of the disease, treatment was administered to asymptomatic persons who had positive xenodiagnosis results who provided consent for being treated. Depending on the availability of drugs, patients were given NIF (8–10 mg/kg/day for 45–60 days, 18 patients) or BZL (5 mg/kg/day for one month, half doses during the first week, 12 patients). None of the patients were pregnant or had associated severe diseases (systemic infections, cardiac, respiratory, renal or hepatic insufficiency, hematologic diseases, and neoplasms) that precluded treatment with NIF or BZL.

Procedures were followed in accordance with the ethical standards of the National Ministry of Health, National Littoral University, and International Guidelines regulating human research. Participants were informed of the study purposes and provided informed consent to participate. Treatment priority to treat patients with firm evidence of circulating parasites was a regulation established by the National Program for the Control and Treatment of chagasic patients.

Xenodiagnosis. Xenodiagnosis was performed by using four entomologic boxes per patient, each containing 10 nymphs III of *Triatoma infestans* that had been starved for three weeks. The boxes were applied to the arms and forearms of patients for 30 minutes. The intestinal content of the insects was analyzed 30, 60 and 90 days after the application. Xenodiagnosis was performed periodically every two or three follow-ups.

Expression and purification of recombinant TcP2β protein. A *T. cruzi* trypomastigote cDNA clone encoding TcP2β protein was subcloned in pET 32a (Novagene, Merck, Darmstadt, Germany) and expressed in the BL21 (DE3) *Escherichia coli* as described.[20] The pET32a vector directs the synthesis of the foreign polypeptides in *E. coli* as fusion polypeptides with the 20.5-kD thioredoxin protein and a histidine tag to facilitate purification of proteins. The 33-kD expressed fusion protein was purified by using NiNTA resin (Qiagen, Valencia, CA) according to the manufacturer's specifications. Briefly, cultures were induced to express protein for three hours with 1 mM isopropyl β-D-1-thiogalactopyranoside, sonicated, and centrifuged for 30 minutes at 4,500 × *g* and 4°C. The supernatants were passed through a NiNTA column. The column was then washed with 50 mM NaH_2PO_4, pH 8, 300 mM NaCl, 50 mM and 100 mM imidazole buffer and eluted with the same buffer plus 250 mM imidazol. Purity of the recombinant protein was analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis[21] and staining with Coomasie blue. Protein quantification was determined by using the Bradford assay.[22]

Antibody assay. Serum levels of antibodies to P2β IgG were measured by using an ELISA. Briefly, microtiter plates (Costar, Cambridge, MA) were coated with 0.5 μg TcP2β in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% bovine serum albumin and incubated with a 1:100 dilution of human serum. The plates were washed and peroxidase-conjugated goat anti-human IgG (Sigma, St. Louis, MO) was added. All incubations were performed at 37°C for 60 minutes. 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 plate, reactive and non-reactive serum samples for each antigen (positive and negative controls, respectively) were assayed simultaneously. The between-plate variation coefficient was < 10%.

Statistical analysis. Comparisons among groups were made in relation to age, sex, distribution of risk factors, antibody levels, and heart involvement. Categorical variables were analyzed by using the chi-square test or Fisher's exact test when unrelated, and the McNemar test was used for analyzing related samples. The Wilcoxon signed-rank test and the Mann-Whitney U test were used to evaluate differences in quantitative values for related and unrelated samples, respectively. A partial correlation coefficient was also calculated. A P value < 0.05 was considered significant.

RESULTS

Clinical findings. The study sample was composed of 78 seropositive persons distributed in three groups. The three groups were composed of 30 asymptomatic persons given NIF (n = 18) or BZL (n = 12) because of the presence of circulating parasites (group A), 37 asymptomatic persons who were not treated (group B), and 11 persons with CCC who were not treated (group C). There were no significant differences between groups A and B groups for age, sex, presence of associated cardiovascular risk factors, and length of follow-up (Table 1).

Most patients from groups A and B remained asymptomatic during the study period (G0), except for five patients in group B in whom ECG abnormalities developed ($P = 0.059$ in comparison with treated patients, by Fisher's exact test). Among patients with CCC, six remained in the same classification, whereas the remaining patients showed evolution to more severe heart involvement ($P < 0.05$, by McNemar test). Three patients had heart enlargement and changes in ECG characteristics (progression from G1 to G2); two other patients showed heart involvement, ECG alterations, grade II heart enlargement, and symptoms of cardiac failure (G1 to G3).

Results of xenodiagnosis were positive in patients in group A. Specific treatment resulted in all patients later showing negative results (an average of four tests/patient was performed). Seven patients in group B and two patients in group C showed positive results.

The most frequent adverse effects of NIF were anorexia, loss of weight, psychological alterations, sleepiness, and digestive manifestations, such as nausea. Adverse reactions caused by BZL were hypersensitivity dermatitis with cutaneous eruptions, edema, and erythema. Skin reactions developed 10–15 days after initiation of treatment and resolved without sequelae.

Antibodies against P2β. The humoral response against *T. cruzi* P2β antigens was analyzed. Serum samples were evaluated at 2–4 different times of patient follow-up for group A (n = 94), group B (n = 76), and group C (n = 44). A decrease in the levels of antibodies to P2β was seen in patients from group A. Final values (mean ± SD = 1.08 ± 0.8) were significantly lower than initial values (1.80 ± 1.29) ($P < 0.0006$) (Figure 1A). Differences were significant regardless whether NIF ($P < 0.0025$) or BZL ($P = 0.009$) were given. Results indicated that a 50% decrease in antibody levels from initial levels occurred in a mean time of 9 years. Patients in groups B and C showed no differences in levels of antibodies to P2β at admission (2.95 ± 1.29 and 1.87 ± 0.96, respectively) and at the end of the follow-up (2.81 ± 1.36 and 1.61 ± 1.04, respectively) (Figure 1B and 1C).

Comparisons between groups were also made. Initial samples from patients in the group A showed lower levels of antibodies to P2β than samples from patients in groups B and C. However, these differences were not statistically significant. However, at the end of the follow-up, lower levels of these antibodies were seen in patients in group A than in patients in group B ($P < 0.0001$, by Mann-Whitney U test). Patients in group B also showed increased levels of antibodies to P2β than in patients in group C with CCC, but this result was not statistically significant ($P = 0.06$).

Levels of antibodies to P2β in the five patients in whom cardiac symptoms developed during the follow-up were initial = 1.68 ± 0.64 and final = 2.12 ± 0.92. For 32 patients who remained asymptomatic, these levels were initial = 2.69 ± 1.72 and final = 2.55 ± 1.54. Statistical comparisons with the Wilcoxon test for related samples showed significant differences in the five patients with cardiac symptoms ($P = 0.04$) but no differences in the 32 patients who remained asymptomatic ($P = 0.99$).

For a better appreciation of changes, we also calculated the relative change in antibodies to P2β by using the formula ([levels at the end of follow-up/levels at the beginning] × 100). Patients undergoing specific treatment (group A) had a relative decrease in antibody levels (75.4 ± 32.7). This finding was not observed in patients in group B (112.6 ± 44.3) ($P < 0.0005$, by Mann-Whitney U test. Further analysis of antibodies to P2β in relation to the clinical evolution of patients with cardiac symptoms showed an increase in four of 10 patients who had aggravation. However, no changes were observed in six patients in whom CCC remained stable ($P < 0.05$, by McNemar test).

Analysis of antibodies to P2β in patients with non-chagasic heart disease was also performed. Patients with CCC had

TABLE 1

Clinical features of the study population, Argentina*

Group	Sex, F/M	Mean ± SD age, years†	CRF	Mean ± SD follow-up, years†	Initial classification	Final classification
Asymptomatic, treated	22/8	29.3 ± 8.6	16	25.1 ± 6.6	G0 = 30	G0 = 30
Asymptomatic, not treated	26/11	32.2 ± 9.8	18	24.1 ± 5.7	G0 = 37	G0 = 32 G1 = 5
With CCC (overall)	5/6	35.4 ± 8.1	3	20.2 ±7.9	G1 = 11	G1 = 6 G2 = 3 G3 = 2

* CRF, cardiovascular risk factors such as smoking, hypertension, and alcoholism; G0 = normal electrocardiograms (ECGs) and chest radiographs; G1 = no congestive heart failure, but ECG showing any of following alterations: complete right bundle branch block and left anterior fascicular block in persons less than 50 years of age, right bundle branch block plus left anterior fascicular block, frequent ventricular extrasystole, ventricular extrasystole associated with conduction disorders, second-degree atrioventricular block, complete atrioventricular block, electrical inactivation areas (no antecedents of ischemic cardiopathy), and a chest radiographic cardiothoracic ratio < 0.50; CCC = chronic chagasic cardiomyopathy; G2 = pathologic ECG tracings, an abnormal chest radiographic cardiothoracic ratio > 0.50, and conserved functional capacity for light and moderate exercises; G3 = congestive heart failure, pathologic ECG tracings, and a chest radiographic cardiothoracic ratio > 0.50. Comparison within the third group (initial 11 G1, final 6 G1, 5 evolved), $P < 0.05$, by McNemar test.

† Age at the time of inclusion.

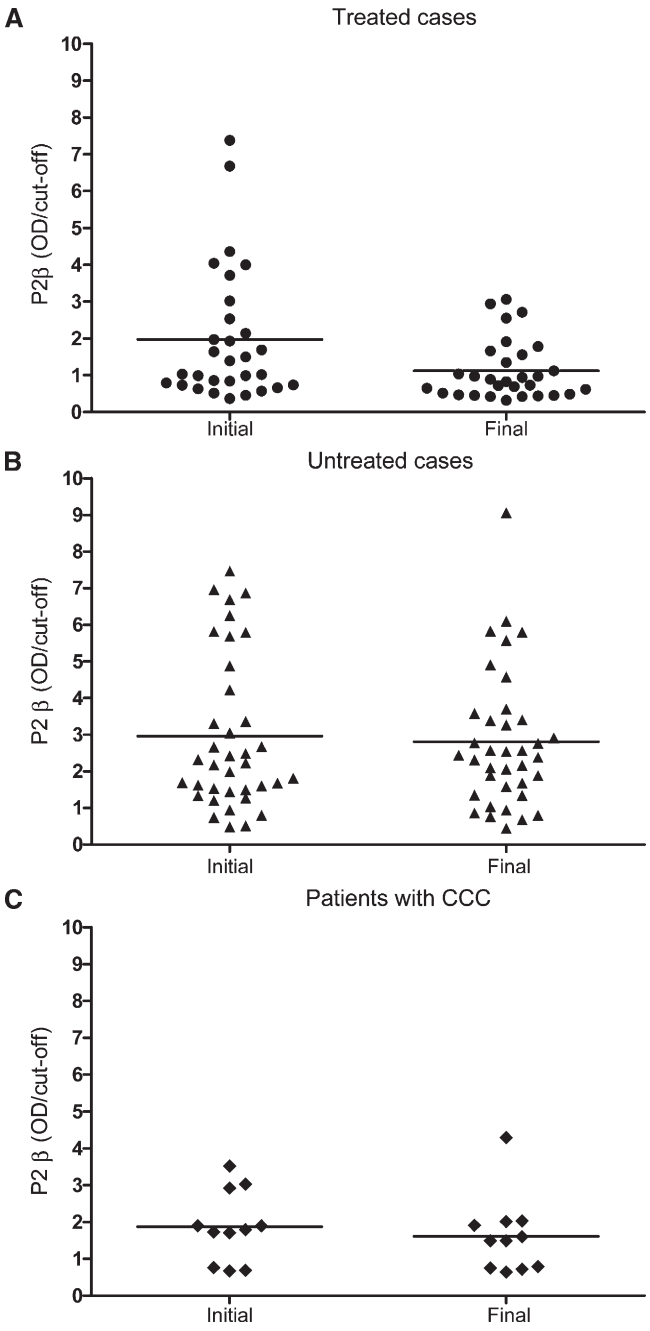

FIGURE 1. Levels of antibodies to P2β at the time of inclusion (initial) and at the end of the follow-up (final) in seropositive persons, Argentina. **A**, **B**, and **C** correspond to treated, untreated, and persons with chronic chagasic cardiomyopathy (CCC), respectively. The y-axis values represent an index between the optical density of the sample/cutoff value. The line represents the mean value. Within group comparisons, final values of treated patients were significantly different from their initial levels ($P < 0.0006$, by Wilcoxon signed-rank test). Between group comparisons in final levels, treated patients versus untreated patients ($P < 0.0001$, by Mann-Whitney U test) and untreated patients versus patients with CCC ($P = 0.06$).

significantly higher levels of antibodies to P2β (mean ± SD = 2.49 ± 1.80) than in non-chagasic patients, in whom levels of antibodies to P2β were scarcely detectable; only two of the 16 patients had values above the cutoff level (0.64 ± 0.06; $P = 0.00001$ compared with CCC cases, by Mann-Whitney U test). Antibodies to P2β were not found in samples from controls.

TABLE 2

Levels of specific antibodies to *Trypanosoma cruzi* in study groups (indirect hemagglutination), Argentina*

Groups	Initial level, mean ± SD†	% Seropositive	Final level, mean ± SD†	% Seropositive†
Asymptomatic, treated	7.07 ± 0.78	100	3.97 ± 1.52‡	46.6§
Asymptomatic, not treated	6.94 ± 0.84	100	6.59 ± 1.01	100
With CCC	7.12 ± 1.26	100	7.19 ± 0.75	100

* CCC = chronic chagasic cardiomyopathy.
† Values are the mean ± SD of the $\log_2$ of the inverse of dilution (representative findings from indirect hemagglutination test). Serum samples were considered reactive when titers were ≥ 1:32 for direct agglutination, indirect hemagglutination, and indirect immunofluorescence.
‡ Statistically significant when compared with initial values ($P < 0.0001$, by Wilcoxon signed-rank test).
§ Significantly different from the two remaining groups ($P < 0.0001$, by chi-square test).

Changes in specific serologic results. Changes in conventional serologic results were also analyzed. In patients in group A, 16 of 30 showed negative serologic results, seven showed significant reduction of antibody titers (doubtful reactions), and seven patients had no changes. Decreased levels of antibodies to *T. cruzi* were seen in either NIF- or BZL-treated patients ($P < 0.0001$ for both drugs, by Wilcoxon signed-rank test) (Table 2). No changes in the serum titers of specific antibodies were seen throughout follow-up in patients in groups B and C (Table 2), which suggested that a higher parasite load not detectable by conventional parasitologic methods (i.e., xenodiagnosis) persisted in these patients. Although conventional serologic tests results and levels of antibodies to P2β showed a similar trend of group differences (i.e., a treatment-related decrease). However, the pairwise correlation did not show a statistically significant difference ($P = 0.29$, by Spearman test).

DISCUSSION

Our study included patients in the late chronic phase of Chagas disease (i.e., persons with infections for greater than 10 years). Treatment priority was given to persons with indeterminate findings with evidence of circulating parasites.

Consistent with findings in other reports[23–25] and extending our earlier findings,[26] treatment for infection with *T. cruzi* decreased specific antibody production, and in some patients led to a persistent negative serologic results and reduced disease progression. In addition, results of xenodiagnosis also became negative in persons treated with NIF or BZL. This finding was not observed in asymptomatic patients not receiving treatment.

Although negative results for xenodiagnosis does not indicate an absence of circulating parasites, results are indicative of a lower parasite load. In addition, treatment with NIF or BZL caused a significant reduction of the antibody response to P2β, which suggests that synthesis of antibodies to P2β is related mostly to the presence of *T. cruzi* antigens. Despite consistency between serologic results and levels of antibodies to P2β, there was no correlation between both sets of data, which suggested that different mechanisms are implied in both phenomena.

Studies in *T. cruzi*-infected animals treated with BZL indicated a direct association between parasite load and the amount of autoreactive responses, as shown by reduced parasitemias, myocardial damage, and autoimmune-associated antibody production.[27,28] As a mutual and non-exclusive possibility, it may

be envisaged that parasite-associated myocardial lesions and subsequent release of self antigens also play some contributory role in production of these antibodies. Data from our series of patients with CCC support this assumption because levels of antibodies to P2β in patients with aggravated cardiac symptoms were higher than those in patients with stable disease.

Against this background, it may be argued that overall levels of antibodies to P2β in patients with CCC remained slightly lower than values in asymptomatic patients. Such disparate results may be reconciled by assuming the existence of two different processes of production of antibodies to P2β. In the first process, antibody production is linked to parasite load and accounts for the overall difference among groups. In the second process, which is more likely to occur in patients with CCC, antibody production is preferably associated with the pathologic state wherein variations in chronic myocardial inflammation and presence of cross-reactive antigens are the main driving forces for antibody production. Results in patients with CCC showed that myocardial damage progressed even when circulating parasites were not noticeable by xenodiagnosis, although presence of parasites below the level of detection of xenodiagnosis cannot be ruled out.

Our results differ from those of studies that reported intense antibody reactivity to P2β in chagasic patients with active myocarditis or severe myocardial involvement.[29,30] Variations in characteristics of patients and/or infecting parasites may account for these differences. Several lines of evidence implicate that parasite strains and persistence, genetic and nutritional features of patients, and the quality of the immune response are involved in the establishment of CCC.[31–33]

Results of our longitudinal study do not lend support to the assessment of antibodies to P2β as a surrogate marker of progression from indeterminate disease to heart disease. However, a slight but statistically significant trend to increased antibody reactivity to P2β was seen in patients with evolving CCC. Further studies in a larger sample of patients, particularly those with cardiac symptoms, are required to ascertain whether this procedure constitutes a useful tool for the diagnosis of disease evolution or worsening of disease.

Quasi-experiments are experiments that include treatments, outcome measures, and experimental units, but do not use random assignment to create comparisons from which effects of treatment can be inferred. Assignment is based on whether a value in a patient becomes higher or lower than a specified value. Because of lack of randomization, groups can differ with regard to other factors, for which differences in outcomes can represent a mixture of effects of the intervention and the effects of such pre-existing differences between groups. In our study, there was acceptable matching between both groups of asymptomatic patients, which significantly reduced the chance of an unbiased estimation of intervention effects.

With regard to the well-known categorization of randomized clinical trials into effectiveness or efficacy trials, Schwartz and Lellouch[34] also proposed a classification into pragmatic and explanatory trials. Pragmatic trials assess effects of an intervention under the usual condition to be applied. Explanatory trials determine the effects of an intervention under ideal circumstances. Findings in our indicate to a great extent what happens in actual clinical situations, which provides further support for specific treatment for chronically infected persons.

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