ARTIGO ORIGINAL

ANTI-M2 MUSCARINIC RECEPTOR AUTOANTIBODIES IN *Trypanosoma cruzi*-INFECTED PEDIATRIC PATIENTS TREATED WITH BENZNIDAZOLE

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ABSTRACT

The presence of autoantibodies with adrenergic and cholinergic activity, capable of triggering neurotransmitter receptor-mediated effects, has been associated to pathogenesis in *T. cruzi*-infected hosts. We determined the presence of anti-M2 muscarinic receptor IgG autoantibodies in 14 pediatric patients with Chagas disease and 18 non-infected infants. *T. cruzi*-infected children showed a higher frequency and a 6.2 ± 1.8-fold increase in the level of autoantibodies to cardiac receptors when compared to controls. Patients were monitored since the initiation of specific treatment with benznidazole (Bz). Along the follow-up, we verified a significant linear decreasing trend in autoantibody reactivity. Remarkably, when treated children became seronegative for *T. cruzi* as direct consequence of parasiticidal chemotherapy, they displayed autoantibody titers similar to those detected in healthy subjects. We conclude that, in pediatric patients, the M2 muscarinic receptor autoantibody response is elicited early in the course of *T. cruzi* infection and decreases after specific treatment, implying that specific Bz treatment eliminates the parasite and reduces potentially pathogenic autoimmune responses.

KEYWORDS: *Trypanosoma cruzi*. M2 muscarinic receptor autoantibodies. Benznidazole. Pediatric patients. Cardiomyopathy.

INTRODUCTION

Chagas´disease, caused by the protozoan parasite *Trypanosoma cruzi*, constitutes an important public health problem in Latin America (WHO 1999). This parasitosis is characterized by an acute phase lasting for 30-60 days, followed by an indeterminate or undifferentiated stage with apparently absent morbidity and, in

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around 30% of infected patients, a chronic stage showing heart or digestive disorders (Elizari 1999). Cardiac manifestations include abnormalities of the intraventricular conduction system, ventricular arrhythmias, sinus node dysfunction, heart failure, aneurysm, and enlargement and dysfunction of the heart (Hagar & Rahimtoola 1995). Arrhythmias can be related with cardiomyopathy by itself or with autonomic nervous system alterations (Milei & Storino 1994). Heart failure and sudden death are the most common causes of decease in patients with Chagas´disease (Hagar & Rahimtoola 1995; Manzullo & Chuit 1999).

Although clinical signs and symptoms appear several years after infection, pathogenic mechanisms can begin since early stages. Several hypotheses have been proposed to account for pathogenesis in chronic *T. cruzi* infections, including parasite persistence in the myocardium (Tarleton 2001), autoimmunity events (Leon et al. 2001) and tissue injury due to exacerbated inflammatory reactions (Rocha et al. 2003). Among alterations induced for host self response, there are data supporting autoantibody production, able to interact with β_1 adrenergic and muscarinic cholinergic M2 receptors in cardiac tissue (Borda & Sterin-Borda 1996), leading to early autonomic dysfunction (Ribeiro et al. 2001). Ferrari et al. (1995) suggested that the presence of these antibodies could be due to molecular mimicry between human β-$_1$ adrenergic / M2 muscarinic receptors and C terminal regions of ribosomal proteins of *T. cruzi*. Recently, this group also demonstrated direct binding of antibodies developed by chronic chagasic patients to the native human β_1 adrenergic receptor (Labovsky et al. 2007).

Andrade et al (1998) found a high frequency of early electrocardiographic alterations in seropositive children from endemic areas, indicating rapid progression from infection to disease. Correa de Araujo et al (1985) observed serious electrocardiographic, radiologic, and anatomo-pathologic abnormalities in children and adolescents with chronic Chagas' heart disease but, unlike adults, they exhibited a rapid evolution to sudden death.

The present study was undertaken to further explore the human M2 muscarinic receptor antibody response in children subjected to trypanocidal chemotherapy with benznidazole in the search for markers of disease progression in pediatric patients with *T. cruzi* infection.

MATERIAL AND METHODS

Population studied

The prospective follow-up study, comprised 14 *T. cruzi*-infected patients, whose ages ranged from 2 months to 8 years (mean: 1.2 years), admitted to the Parasitology Service of Ricardo Gutiérrez Children Hospital at Buenos Aires, Argentina, for their diagnosis and treatment. They were recruited in a lapse of 3

years, between 2003 and 2006. Age- and sex-matched children (n=20) seronegative for *T. cruzi*, were considered as controls. This study was approved by the Institutional Review Board.

Diagnosis criteria

In children ≥ 7 months of age, the serologic diagnosis of *T. cruzi* infection was carried out by indirect hemagglutination (IHA, Lab. Polychaco, Buenos Aires, Argentina), enzyme-linked immunosorbent assay (ELISA, Wiener, Rosario, Argentina) and passive particle agglutination test (PPA, Bayer, Buenos Aires, Argentina). IHA and PPA antibody titers > 16, as well as ELISA positive ratios higher than 1.2, were considered reactive. Infants with two or more positive tests were diagnosed as seroreactive to *T. cruzi*. In children < 7 months of age, parasitologic diagnosis of *T. cruzi* infection was performed by microhematocrit test (MH) as previously published (Freilij et al. 1983).

Therapeutic regimen and treatment follow-up

All *T. cruzi*-infected infants were treated with benznidazole (Bz, RADANIL®, Roche, Argentina) at 5-8 mg/kg/day in 2-3 daily oral doses, for 60 days. Since the beginning of treatment (T0), patients were followed up by clinical (electrocardiogram-ECG- and echocardiogram-ECHO) and laboratory (MH, IHA and ELISA; hemogram, hepatogram, creatinin) evaluations at 30 days, 60 days (end of treatment) and 6 months (T1) after completion of chemotherapy. Additionally, another sample was collected at the end of follow-up (T2, mean =23.7 months, range: 18 to 36 months), defined by negativization of conventional serology in two consecutive samples.

Measurement of anti-autonomic neurotransmitter antibodies

Serum samples, obtained at T0, T1 and T2 of follow-up, were stored at -20ºC until measuring anti-M2 muscarinic receptor IgG autoantibodies by a commercial ELISA kit according to manufacturer´s instructions (Chagacor, Lab. Lemos S.R.L, Buenos Aires, Argentina).

Statistical analysis

Statistical analyses were carried out using the Prisma 4.0 software (GraphPad, San Diego, CA, USA). Fisher´s exact test, one-way ANOVA or Student´s t tests were performed as appropriate. *P* values of <0.05 were considered significant.

RESULTS

Thirty-two children included in our study were examined for the presence of neutrotransmitter autoantibodies. At diagnosis, *T. cruzi*-infected patients showed an extremely (p=0.0008) higher frequency of these serum autoantibodies than that determined in control subjects (64.3% vs. 11.0 %, respectively). Furthermore, we observed a 6.2 ± 1.8 fold increase in the level of antibodies to cardiac receptors in children with Chagas infection when compared to controls (Figure 1).

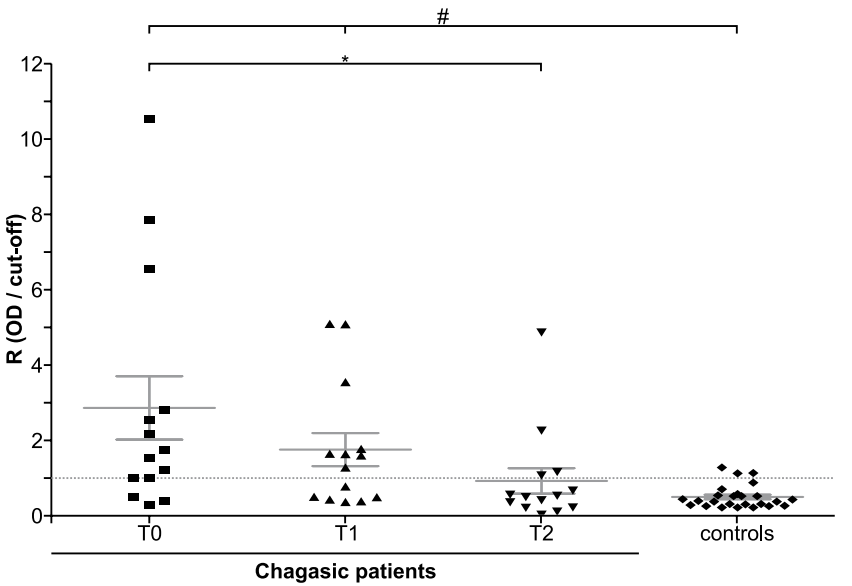

Figure 1. Distribution of anti-neurotransmitter antibodies in sera of pediatric chagasic patients. Sera from 14 patients with Chagas´disease and 20 uninfected controls were tested by ELISA for anti-M2 muscarinic receptor IgG autoantibodies. T0: beginning of treatment, T1: 6 months after completion of chemotherapy, T2: end of follow-up (patients serologically negative for *T. cruzi*-specific IgG antibodies). Each point represents one patient. The group mean and the standard error of the mean are also depicted. Dotted line shows cut-off level of the assay. R, ratio between optical densities (OD) determined for each serum sample and cut-off value. * p<0.05, T0 *vs* T2; # p<0.05, T0 and T1 *vs* controls.

T. cruzi-infected infants were monitored since the initiation of chemotherapy. Along the follow-up, we verified a significant (p=0.02) linear decreasing trend in their autoantibody titers. As shown in Figure 1, Chagas patients sera collected at T0 and T1 presented higher (p<0.05) reactivity than samples from

healthy controls. At the end of survey period (T2), ELISA values determined in Bz-treated patients were lower ($p<0.05$) than those detected at T0, and did not differ from those found in the non-infected population.

No correlation between autoantibody response and heart dysfunction could be established in pediatric Chagas patients, as all children presented normal ECG and ECHO examinations.

DISCUSSION

Autoantibodies with adrenergic and cholinergic activity, capable of triggering neurotransmitter receptor-mediated effects, have been demonstrated in human (Goin et al. 1999) and murine (García et al. 2005) chagasic sera. Even though elevated autoantibody levels have also been linked to cardiomyopathies distinct from Chagas disease, no induction could be verified in other parasite infections. This kind of self-reactive response may promote several biochemical and functional alterations on normal myocardial activity (Goin et al. 1994; Goin et al. 1999). Nevertheless, whether the autoantibodies are a cause or a consequence of disease still remains unclear. It has been suggested that the generation of autoantibodies to cardiac receptors is due to molecular mimicry phenomena occurring during the course of *T. cruzi* infection (Masuda et al. 1998), rather than actual lymphocyte-mediated autoreactivity. Regardless of their origin, the presence of anti-cardiac immunoglobulins definitely contributes to Chagas pathogenesis in the infected host. Indeed, these autoantibodies are observed with higher frequency in adults with chronic Chagas heart disease than in asymptomatic or indeterminate *T. cruzi*-infected patients, suggesting that self-reactive antibodies could be used as a premature marker of evolution for Chagas cardiomyopathy (Talvani et al. 2006; Giménez et al. 2003).

Our present work constitutes the first report demonstrating the induction of anti-neurotransmitter receptor autoantibodies in pediatric patients at early stages of *T. cruzi* infection. In addition, the analysis of serum specimens collected before and after treatment with Bz, the main drug available for the treatment of Chagas´ disease, showed a clearly decreasing trend in autoantibody levels. Remarkably, at the time of follow-up when treated patients became seronegative for *T. cruzi* as direct consequence of trypanocidal chemotherapy, they presented autoantibody titers as low as those displayed by healthy subjects. The beneficial effects of Bz treatment in *T. cruzi* infection may not only depend on its parasiticidal effect but also on immunomodulating influences. Piaggio et al. (2001) demonstrated that the production of immune response mediators, like nitric oxide and cytokines, was modified by specific chemotherapy. Importantly, changes in the levels of tissue-reacting antibodies were detected in nifurtimox-treated patients with acute Chagas´ disease (Schmuñis et al. 1978). Our results indicate that anti-neurotransmitter receptor autoantibodies in pediatric Chagas patients may also be downregulated after treatment with Bz.

A recent study in the murine model of chronic *T. cruzi* infection demonstrated that therapy with Bz not only diminished the parasite burden but also prevented the development of ECG alterations. Moreover, sera of Bz-treated mice had lower levels of antibodies against β_1-adrenergic and M2-muscarinic cardiac receptors than the sera of the untreated group (García et al. 2005).

We attempted to search for any correlation between anti-cardiac autoantibodies and heart dysfunction in our pediatric patients with *T. cruzi* infection. In contrast with the findings of Andrade et al (1998), we were unable to detect ECG or ECHO abnormalities. This discrepancy might be attributed to the size and characteristics of the populations evaluated for each study. In that cross-sectional survey, larger samples of both infected and control groups were examined. A total of 141 *T. cruzi* seropositive and 282 seronegative children (mean age, 10.4 years) from an endemic area in Brazil were subjected to electrocardiographic studies, finding a prevalence of ECG alterations of 11.3% among seropositive children and 3.5% among seronegative children. On the other hand, our population had a smaller size and was constituted by much younger (mean age, 1.2 years) pediatric patients living in non-endemic regions of Argentina.

We conclude that, in pediatric patients, the M2 muscarinic receptor autoantibody response is elicited early in the course of *T. cruzi* infection, and decreases after trypanocidal chemotherapy, implying that specific Bz treatment eliminates the parasite and reduces potentially pathogenic autoimmune responses.

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RESUMEN

Autoanticuerpos contra receptores muscarínicos M2 en pacientes pediátricos infectados con *Trypanosoma cruzi* y tratados con benznidazol

La presencia de autoanticuerpos con actividad adrenérgica y colinérgica, capaces de modificar la actividad de los receptores a neurotransmisores, ha sido asociada a la patogénesis de la Miocardiopatía chagásica. Hemos investigado la existencia de autoanticuerpos contra receptores muscarínicos M2 en la fracción IgG de 14 pacientes pediátricos con Enfermedad de Chagas y en 18 controles no infectados. Encontramos una mayor frecuencia y un incremento de 6,2 ± 1,8 veces en el nivel de autoanticuerpos contra receptores cardíacos en los niños infectados con *T. cruzi* respecto de los controles no infectados. Luego del tratamiento parasiticida específico, los pacientes fueron evaluados prospectivamente, comprobándose una tendencia lineal descendente significativa en la reactividad de estos autoanticuerpos.

Al producirse la seroconversión negativa para *T. cruzi* como consecuencia directa del tratamiento, los pacientes presentaron títulos de autoanticuerpos contra receptores cardíacos similares a los detectados en los niños no infectados. Concluimos que en pacientes pediátricos la respuesta de autoanticuerpos contra receptores muscarínicos M2 se manifiesta tempranamente en el curso de la infección con *T. cruzi* y decrece después de la quimioterapia especifica. Por lo tanto, la administración de BZ a estos pacientes no sólo sería efectiva para eliminar el parásito sino también para reducir respuestas autoinmunes potencialmente patogénicas.

DESCRITORES: *Trypanosoma cruzi*. Autoanticuerpos contra receptores muscarínicos M2. Benznidazol. Pacientes pediátricos. Cardiomiopatía.

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