Immunological Assessment of Benznidazole Therapy in Chronic Chagas Disease

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ABSTRACT

To determine the effect of benznidazole therapy on memory T cells specific for *Trypanosoma cruzi*, 47 patients between 30 and 50 years old and three positive serological tests for *T. Cruzi* without heart involvement were selected. Benznidazole was administered in a dose of 5 mg/kg/d during 30 days. Serological, immunological and clinical assessment was performed at basal (time 0) and at 2, 6 and 12 months following treatment, and once a year thereafter. IFN-γ ELISPOT assay was used to evaluate T lymphocytes response against a *T. cruzi* lysate obtained from amastigotes. The frequency of IFN-γ -producing memory T lymphocytes specific for *T. cruzi* was significantly lower in the treatment group (n=33) compared to the control group (n=14) 12 months after the therapy. IFN-γ response became negative in 11 patients in the treatment group (44%). Among these 11 patients, conventional serology also became negative in 4 patients (36%) after 2 years of treatment. No clinical manifestations occurred during follow-up. These findings show that benznidazole is capable of modulating T lymphocytes response specific against *T. cruzi*. Measuring the frequency of memory T lymphocytes producing IFN-γ might become a more sensitive test to determine earlier the impact and/or efficacy of the specific treatment against this parasite.

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Key words > Chagas disease - Benznidazole - Lymphocytes - *Trypanosoma cruzi*

Abbreviations >

HIV Human immunodeficiency virus	**IL-2** Interleukin-2
IFN-γ Gamma interferon	

BACKGROUND

Chagas disease is a parasitic condition caused by a flagellate protozoan, *Trypanosoma cruzi*. This parasite can be transmitted to humans through different ways: vectorial, congenital (from a pregnant woman to her baby) through the placenta, blood transfusions, organ transplantation and probably digestive transmission. *T. cruzi* produces parasitemia, invades cells, makes nests and multiplies in tissues leading to tissue inflammation and destruction. The organs particularly involved are the heart, digestive system and nervous system. In Central America and South America there are more than 10 million people infected. (1)

Approximately 30% of infected persons develop chronic cardiomyopathy after an indeterminate period of one or two decades after the initial infection. During this period host and parasite share a stable coexistence and there is no clinical evidence of the disease as host's immunological system controls the infection managing parasite replication. Occasionally, this stability gets lost due to presence of concomitant diseases, administration of immunosuppressive drugs or by exhaustion of the immune system; thus the infection is not adequately controlled leading to clinical manifestations. Symptoms include arrhythmias, conduction disorders, heart enlargement, congestive heart failure, thromboembolism and sudden death. (2)

Immune response is critical to control the parasite due to its intracellular location. Antigen presenting cells take up antigens from the blood, process them and present them to T lymphocytes in the lymph nodes. These lymphocytes recognize the antigen, proliferate and differentiate in different cellular populations. Effector CD4 and CD8 T lymphocytes migrate to infected tissues where they release IFN-ã which activates macrophages. Effector CD8+ T lymphocytes are also capable of producing lysis of parasitic cells. Memory T cells comprise two subtypes: central memory T cells and effector memory T cells. Central memory T cells population develop only after clearance of the antigen and releases IL-2. Conversely, effector memory T cells population is only

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present if antigen persists and has the particularity to produce IFN-γ in the presence of new exposure to antigens. This study was performed on the population of T. cruzi-specific effector memory T cells.

Chagasic cardiomyopathy evolves slower compared to other dilated cardiomyopathies. (49 The physiopathological mechanism of the disease is controversial due to the need for long-term follow-up of patients, the low number of parasites in blood and to the persistence of circulating antibodies even after the parasite has been cleared. (4) Nevertheless, there is evidence that intracellular persistence of *T. cruzi* in the myocardium is the stimulus for chronic inflammatory response that leads to tissue damage. (5, 6) It has been demonstrated that antiparasitic treatment is effective in the acute stage of the disease. (7, 8) However, several reasons limit its use during the chronic stage. (9-13) The main limitations in the assessment of treatment for chronic Chagas disease arise from the need for long-term follow-up to detect serological cure, and the lack of reliable tests to ensure elimination of the parasite. (14) Little is known about the impact of specific chemotherapy against T. cruzi infection on host's immune response. (15, 16)

We have previously demonstrated that patients without heart disease or with mild heart involvement present greater levels of parasite-specific effector memory T lymphocytes, compared to the levels observed in patients with advanced disease. (17) About 80% of asymptomatic patients present positive IFN-ã ELISPOT assays and only 15% of patients with chronic infection and congestive heart failure display substantial amounts of IFN-ã producing T cells. There is an inverse relationship between the severity of heart diseaer and the number of effector memory T lymphocytes. We consider that patients with severe heart disease are not capable of an adequate immune response due to the fact that their immune system is exhausted. (18) The benefit of long-term therapy with benznidazole has been reported by two clinical studies. (9, 19)

The aim of this study was to determine the effect of benznidazole therapy on *Trypanosoma cruzi*-specific memory T cells in patients in the indeterminate stage of chronic Chagas disease.

MATERIAL AND METHODS

Selection of Study Population

Participants of the present study were selected among patients attending the Section of Chagas Disease, Hospital Interzonal de Agudos "Eva Perón", in the Province of Buenos Aires. T. cruzi infection was determined by indirect immunofluorescence assay, indirect hemagglutination test, and ELISA technique. Patients were grouped according to the Kuschnir grading system. (20)

Inclusion Criteria

Patients were included according to the following criteria: three positive results of serological testing (IHA, IIF and ELISA), age between 21 and 50 years, and absence of any of the following conditions: heart disease of any etiology, hypertension, diabetes, dyslipemia and other diseases such as cancer, arthritis, allergies, or HIV infection, and pregnancy. Patients assigned to the treatment group should have completed the treatment schedule.

Patients eligible for this blind study were randomly assigned to two groups, treatment group and control group, in a 2:1 ratio, respectively.

Treatment Schedule

Patients were treated with oral benznidazole at a maximum dose of 5 mg/kg per day for 30 days after an initial adaptation period of 7 days starting with increasing doses. This is the treatment schedule that we traditionally recommend to our patients (9, 19) based on previous evidence showing no significant differences with therapies lasting longer than 30 days in the efficacy to produce negative seroconversion. Severe adverse events can be prevented if the cumulative dose does not exceed 18 gr of benznidazole. (10)

Characteristics of Study Population

Forty seven patients were finally included (28 women and 16 men). Mean age was 41.64 ± 6.2 years + SD. All participants belonged to group 0 according to the clinical classification of Kuschnir (33 in treatment group and 14 in control group).

Patients Control

Serological, immunological and clinical evaluation were determined at inclusion (time 0) and at 2, 6 and 12 months; routine laboratory tests were performed at 0 and 12 months; ECG were obtained at 0, 2, 6 and 12 months, and chest X-ray, stress test and echocardiogram at time 0. Thereafter, controls were performed once a year. Signed informed consent was obtained from all participants. The protocol was approved by the Committee of Ethics of the Hospital Eva Perón and the University of Georgia.

Collection of Peripheral Blood Mononuclear Cells and Serum

Approximately 50 ml of blood were obtained from each participant by venipuncture into heparinized or nonheparinized vacuum tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood by density-gradient centrifugation. Nonheparinized blood was incubated at 37 °C and allowed to coagulate; then it was centrifugated at 1.000 × g for 15 minutes and serum was separated.

Obtaining Protein Lysate from *T. cruzi* Amastigotes

Protein lysate from *T. cruzi* amastigotes was obtained by freeze/thaw cycles, which were followed by sonication. (17)

ELISPOT Assays

Sterile nitrocellulose 96-well microplates were coated with monoclonal anti–IFN-ã antibodies and incubated at 4 °C for 18 hours; PBMCs were seeded in triplicate, at a concentration of 4×10^5 cells/well and were stimulated with 10 μg/ml of amastigotes lysate or cultured for 16 hours at 37°C in a 5% CO2 environment. (17) Then biotinylated anti–IFN-ã was added and the plates were incubated at room air for 2 hours. Each spot representing IFN-γ producing cell was detected after color development with amino-ethylcarbazol. Spots were counted by an ELISPOT reader system (CTL, USA). Responses were considered to be positive when the number of spots was 10 or greater and when this number was at least twice the value of spots found by use of culture medium. The number of *T. cruzi*-specific T lymphocytes was calculated by subtracting the value of the T lymphocytes

obtained without stimulation from the number of T lymphocytes obtained with the lysate stimulus. (17)

Statistical Analysis

Quantitative changes in cellular response were determined by ANOVA and Friedman test. The Mann-Whitney U test was used to compare changes in variations in cellular response between treatment and control groups on post-treatment/pretreatment differences for each group. Comparisons of cathegories among both groups were evaluated by use of the chi-square test and Fisher's exact test. A p value dH 0.05 was considered statistically significant.

RESULTS

Monitoring of T. cruzi–Specific Effector Memory T Cells Response

In different infections in humans, the presence of IL-2-producing central memory T lymphocytes was associated with resolution of infection, while IFN-γ-producing effector memory T cells are characteristic of persistent infections. (22-24) Monitoring function of T cells constitutes a powerful tool to determine the activity of the disease. In order to evaluate if benznidazole is capable of modulating *T. cruzi*-specific effector memory T cells response in the indeterminate stage of the infection we quantified frequency of IFN-γ -producing T lymphocytes using ELISPOT technique.

The frequency of *T. cruzi*-specific IFN-γ -producing T lymphocytes significantly decreased in the treatment group compared to the control group at 12-month follow-up.

Twenty five out of 33 patients in the treatment group (76%) presented positive ELISPOT assay prior to therapy, whereas the test was negative in 8 patients (24%) (Figure 2). ELISPOT assay became negative in 11 of 25 (44%) patients with a positive result before the therapy (8 patients and 3 patients at 12 and 24 months after treatment, respectively); however, ELISPOT technique did not become negative in any of the patients in the control group, with stable levels of IFN-γ -producing T lymphocytes (Figure 2, Fisher's test between the percentage of treated patients that produced a negative seroconversion of the ELISPOT assay versus the percentage of control patients with a negative assay, $p < 0.05$).

Therapy with benznidazole produced immunological changes in 5 of the 8 patients with negative ELISPOT assay before treatment who presented detectable levels of IFN-γ -producing T lymphocytes during follow-up.

These outcomes suggest that ELISPOT technique might constitute a sensitive and early assay to determine the impact of the specific treatment against *T. cruzi*.

Kinetics of Effector Memory T Cell Response in Patients Treated with Benznidazole

In order to assess kinetics of cellular response, IFN-γ production was determined in a group of 18 patients (12 in the treatment group and 6 in the control group) at 2 and 6 months during follow-up. There was no evidence of a reduction in the production of IFN-γ before the first year after therapy.

Patients in the treatment group presented two types of response: one group showed an increase in the number of lymphocytes followed by progressive reductions in the number prior to negative serocon-

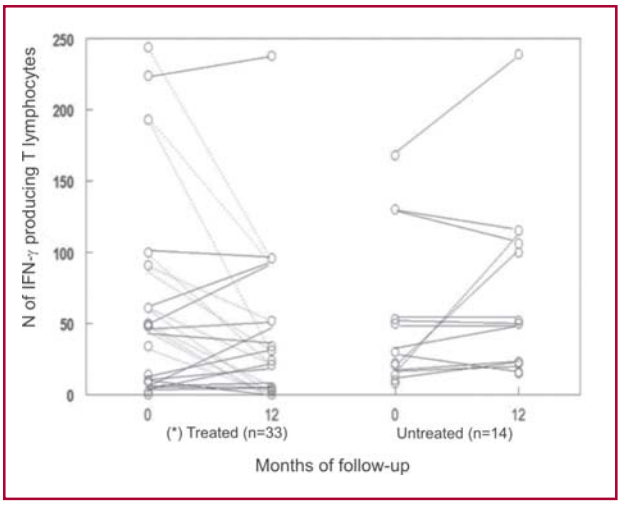

Fig. 1. Effect of treatment with benznidazole on *Trypanosoma cruzi*-specific memory T cell response in patients with chronic Chagas disease. The number of IFN-g producing T lymphocytes specific against T. cruzi was determined by ELISPOT before therapy and 12 months after treatment with benznidazole. Each symbol represents the average number of spots for each patient. (*) p < 0.005 Mann-Whitney U test test used to compare changes in variations in cellular response between treatment and control groups.

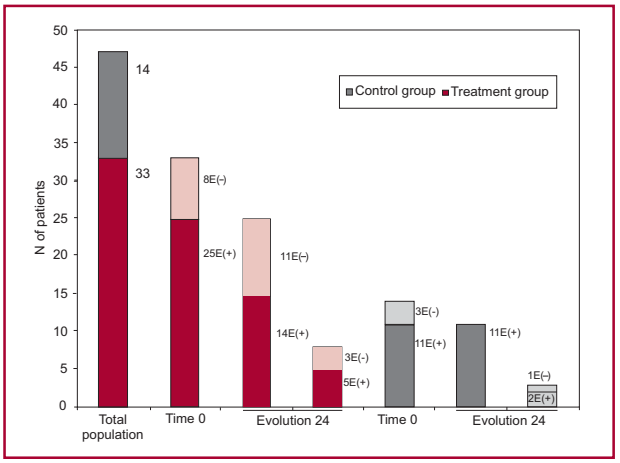

Fig. 2. Evolution of *Trypanosoma cruzi*-specific memory T cell response in patients treated with benznidazole and in untreated patients. (*) Fisher's test p < 0.05 (% of negative seroconversion in the treatment group versus % of negative seroconversion in the control group with basal (+) ELISPOT assay)

version; this increase in the number of lymphocytes was observed between 2 and 6 months after therapy. Another group presented progressive reductions in the response before the negative seroconversion with absence of the peak described in the aforementioned group (Figure 3).

Relationship between ELISPOT and Conventional Serological Tests Responses

Four out of 33 patients in the treatment group (12.12%) presented negative seroconversion of the all three conventional serological tests: 1 at 12 months and 3 at 24 months after therapy. In 4 of the 11 patients (36.26%) in whom IFN-γ ELISPOT response became negative, negative seroconversion of conventional serological tests after therapy was also observed.

DISCUSSION

The infection by *T. cruzi* remains unnoticed in most patients, and only a few of them will develop heart disease several years after the initial infection.

Once heart disease has developed, it is possible to establish a prognosis for disease progression based on clinical indicators; (25) however, the detection of patients who will evolve to cardiomyopathy is still a mystery. There is still an absence of indicators of therapeutic success in patients with chronic infection by *T. cruzi*. (9, 14) Nevertheless, assessment of patients' immunological status against the parasite showed that those patients in better clinical conditions presented greater levels of IFN-γ -producing memory T lymphocytes specific for *T. cruzi* compared to patients with severe heart involvement. (17) When we analyzed patients in group 0 (without heart disease) we found that the immunological behavior was not the same. Most patients presented the same profile of effector memory T cell (producing IFN-ã and IL-2) and absence of IL-2-producing central memory lymphocytes. (26)

Proliferative capacity of central memory T cells is maintained in the absence of antigen and may persist for a long time; in contrast effector memoryT cells have low proliferative capacity and depend on antigen persistence. (27) In addition, the severity of the disease associated with greater frequency of total highly differentiated and apoptotic CD8+ memory T lymphocytes with characteristics of senescence cells, (18) supports the idea that chronic infection by *T. cruzi* leads to exhaustion of the immune system as a consequence of the persistence of the antigen. This might affect the capacity of the immune system to control the infection, leading to progression of the disease.

This research evaluates the impact of therapy with benznidazole in the chronic stage of the infection by *T. cruzi* on immune cellular response specific for this parasite. The results show that etiology-directed therapy against *T. cruzi* may modulate parasite-specific effector memory T cell response. Effector memory T cells depend on the presence of the antigen, thus the decrease in the ELISPOT response for IFN-γ one year after therapy might serve as an early potential

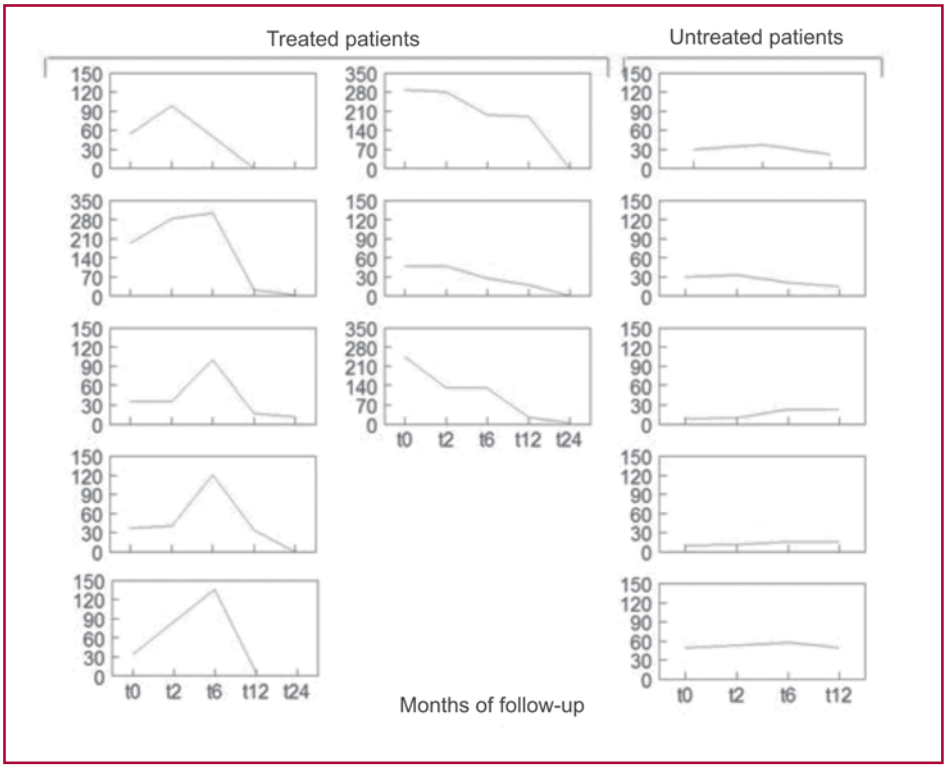

Fig. 3. Kinetics of effector memory T cell response in patients treated with benznidazole. The ELISPOT response for IFN-ã was measured at different periods of time after follow-up in patients treated with benznidazole and in a group of untreated patients. The different plots represent the behavior for individual patients. The frequency of IFN-γ producing T lymphocytes increases between 2 and 6 months of follow-up and then decreases ($p < 0.05$; left column); progressively decreases ($p < 0.05$; central column) or remains unchanged (right column) according to Friedman test.

marker of elimination/reduction of the parasite burden. Follow-up time was not long enough to detect significant changes in conventional serological tests; however, we have noticed that in 4 patients treated with benznidazole conventional serological tests became negative as well as the frequency of IFN-γ producing memory T lymphocytes. This interpretation is also supported by the fact that therapy was capable of modifying serological titration of recombinant *T. cruzi* proteins.

A significant increase in the population of IFN-γ memory T cells at 2 and 6 months after therapy was observed in a high proportion of patients with negative seroconversion of cellular response, indicating a greater release of parasitic antigens due to benznidazole effect, which stimulated cellular response. This might be related with a greater sensitivity to benznidazole or to a greater parasitic burden in these patients prior to therapy. These findings might also explain the increase in ELISPOT response for IFN-γ seen in patients with a negative response before treatment. These results are consistent with previous observations in the murine model as they demonstrate that activation of host's immune system by parasitic antigens and endogenous IFN-γ might play a predominant role in the efficacy of benznidazole treatment. (28 – 30) The association between cellular response and the progression of the disease as a consequence of etiology-related therapy might be assessed at long-term follow-up. The use of benznidazole during the chronic stage of the disease might be justified in case a positive effect of therapy on cellular immune response against *T. cruzi* is found. In addition, this research provides valuable information on the contribution of cellular immune response in parasite control.

In summary, our findings show that benznidazole is capable of modulating memory T cell response specific for *T. cruzi*. Measuring the frequency of IFN-γ producing T lymphocytes might constitute a more sensitive and earlier assay to determine the impact/efficacy of a specific treatment against this parasite.

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RESUMEN

Evaluación inmunológica del tratamiento con benznidazol en la enfermedad de Chagas crónica

Para determinar el efecto del tratamiento con benznidazol sobre las células T de memoria específica para *Trypanosoma cruzi*, se seleccionaron 47 pacientes con tres reacciones serológicas positivas para *T. cruzi*, sin cardiopatía y edades comprendidas entre los 30 y los 50 años. El tratamiento se realizó con benznidazol en dosis de 5 mg/kg/d por 30 días. Se efectuó una evaluación serológica, inmunológica y clínica pretratamiento (tiempo 0) y a los 2, 6 y 12 meses postratamiento. Posteriormente, los controles se hicieron anualmente. La respuesta de linfocitos T frente a un lisado de amastigotas de *T. cruzi* se evaluó por la técnica de ELISPOT para IFN-γ. La frecuencia de linfocitos T de memoria productores de IFN-γ específicos para *T. cruzi* disminuyó significativamente en el grupo tratado (n = 33) *versus* el no tratado (n = 14) 12 meses después del seguimiento. Once de 25 (44%) pacientes que recibieron benznidazol negativizaron la respuesta para IFN-γ. Cuatro de los 11 (36%) pacientes con ELISPOT (+) que negativizaron la respuesta por ELISPOT para IFN-g también negativizaron la serología convencional a los 2 años postratamiento. Durante el seguimiento no se observaron alteraciones clínicas. Estos hallazgos muestran que el benznidazol es capaz de modular la respuesta celular T de memoria específica para *T. cruzi*. La medición de la frecuencia de linfocitos T de memoria productores de IFN-γ podría constituir un ensayo más sensible y precoz para determinar el impacto/eficacia del tratamiento específico contra este parásito.

Palabras clave > Enfermedad de Chagas - Benznidazol - Linfocitos T - Trypanosoma cruzi

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