Changes in *Trypanosoma cruzi*-Specific Immune Responses after Treatment: Surrogate Markers of Treatment Efficacy

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(See the article by Bern et al, on pages 1667–74, and the editorial commentary by Urbina, on pages 1685–7.)

**Background.** As many as 20 million people are living with *Trypanosoma cruzi* infection in Latin American, yet few receive any treatment. The major limitation in developing and evaluating potential new drugs for their efficacy is the lack of reliable tests to assess parasite burden and elimination.

**Methods.** Adults volunteers with chronic *T. cruzi* infection were evaluated clinically and stratified according to the Kuschnir classification. Individuals with group 0 and group 1 clinical status were treated with benznidazole (5 mg/kg per day for 30 days). The changes in *T. cruzi*-specific T cell and antibody responses, as well as in clinical status, were measured periodically over the 3–5-year follow-up period and were compared with pretreatment conditions and with values in an untreated control group.

**Results.** The frequency of peripheral interferon (IFN)-γ-producing T cells specific for *T. cruzi* declined as early as 12 months after benznidazole treatment and subsequently became undetectable in a substantial proportion of treated subjects. In addition, decreases in antibody responses to a pool of recombinant *T. cruzi* proteins also decreased in many of these same subjects. The shift to negative IFN-γ T cell responses was highly associated with an early increase in IFN-γ-producing T cells with phenotypic features of effector/effector memory cells in a subset of subjects. Benznidazole treatment also resulted in an increase in naive and early differentiated memory–like CD8+ T cells in a majority of subjects.

**Conclusions.** Benznidazole treatment during chronic Chagas disease has a substantial impact on parasite-specific immune response that is likely indicative of treatment efficacy and cure.

The most serious long-term sequela of chronic *Trypanosoma cruzi* infection is the development of a persistent inflammatory cardiomyopathy that may lead to congestive heart failure and death. Although therapy with nitroimidazole derivatives is recommended to the Kuschnir classification. Individuals with group 0 and group 1 clinical status were treated with benznidazole (5 mg/kg per day for 30 days). The changes in *T. cruzi*-specific T cell and antibody responses, as well as in clinical status, were measured periodically over the 3–5-year follow-up period and were compared with pretreatment conditions and with values in an untreated control group.

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**Conclusions.** Benznidazole treatment during chronic Chagas disease has a substantial impact on parasite-specific immune response that is likely indicative of treatment efficacy and cure.
ing of the relationship between parasite persistence and the maintenance of *T. cruzi*-specific T cells during chronic Chagas disease by examining the effect of treatment with benznidazole on the frequency, function, and phenotype of general and *T. cruzi*-specific T cells in chronically infected subjects who had been treated with benznidazole 3–5 years previously. We demonstrate that *T. cruzi*-specific T cell responses declined in association with decreases in antibody responses to a pool of *T. cruzi* recombinant proteins from *T. cruzi* and increases in the CD8+ T cell count with early differentiated/antigen-experienced phenotype in a substantial proportion of treated subjects but not in the untreated group.

**METHODS**

**Selection of study population.** *T. cruzi*-infected adults volunteers aged 21–54 years were recruited at the Chagas Disease Section of Hospital Interzonal General de Agudos “Eva Perón” (Buenos Aires, Argentina). *T. cruzi* infection was determined by indirect immunofluorescence assay, hemagglutination, and enzyme-linked immunoassay techniques [23] performed at the Instituto Nacional de Parasitología “Dr. Mario Fatala Chaben” (Buenos Aires). Chronically infected subjects were evaluated clinically and stratified according to the Kuschnir grading system [24]. Individuals in group 0 had normal electrocardiograph, normal chest radiograph, and normal echocardiograph findings (n = 67; mean age, 38.68 years; range, 23–55 years), and subjects in group 1 had normal chest radiograph and echocardiograph findings but abnormal electrocardiograph findings (n = 8; mean age, 43.88 years; range, 32–52 years).

Treatment consisted of benznidazole, 5 mg/kg per day for 30 days [5–7]. Subjects in clinical group 0 were assigned randomly to the treated and untreated group; group 1 patients (n = 8) were all assigned to the treatment group on the basis of previous studies demonstrating clear evidence of the efficacy of treatment on progression of disease in this subject group [5, 7]. Clinical, serological, and immunological analysis was performed before treatment; 2, 6, and 12 months after treatment; and at yearly intervals thereafter.

This protocol was approved by the institutional review boards of the University of Georgia and the Hospital Interzonal General de Agudos “Eva Perón.” Signed informed consent was obtained from all individuals before inclusion in the study.

**Collection of peripheral blood mononuclear cells (PBMCs) and serum specimens.** PBMCs were isolated by density gradient centrifugation on Ficoll-hypaque (Amersham) and were cryopreserved for later analysis. Blood to be used for serum analysis was allowed to coagulate at 4°C and centrifuged at 1000 g for 15 min for sera separation.

**IFN-γ and interleukin (IL)-2 enzyme-linked immunosorbent spot (ELISPOT) assays.** The number of *T. cruzi*-specific IFN-γ– and IL-2–secreting T cells was determined by ex vivo ELISPOT using a commercial kit (ELISPOT Human IFN-γ or IL-2 ELISPOT Set; BD), as described elsewhere [21, 25]. To avoid interexperiment variations, assays were conducted with paired samples from different time points. Each time point was assessed 1–3 times.

**Flow cytometric detection of *T. cruzi* antigen–induced intracellular IFN-γ and T cell phenotyping.** IFN-γ production was determined after stimulation of PBMCs with 15 μg/mL *T. cruzi* lysate or media alone for 16–20 h, with the addition of 10 μg/mL brefeldin A for the last 5 h of incubation, as described elsewhere [22, 25]. The cells were then stained with anti-CD4 (peridin chlorophyll protein) or anti-CD4 (fluorescein isothiocyanate) with the appropriate combination of anti CCR7 (phycoerythrin [PE]), anti KLRG1 (allophycocyanin), anti-CD122 (peridin chlorophyll protein) or anti-CD122 (fluorescein isothiocyanate) with the appropriate combination of anti CCR7 (phycoerythrin [PE]), anti KLRG1 (allophycocyanin), anti-CD122 (PE), anti-CD127 (PE), and IFN-γ (APC) or IFN-γ (PE), all of which were from BD-Pharmingen.

Data were acquired on a FACS Calibur cytometer (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson). Typically, 500,000 events were collected per sample.

**Table 1. Characteristics of Study Population, According to Interferon (IFN)-γ Enzyme-Linked Immunosorbent Spot (ELISPOT) Responses before Treatment with Benznidazole**

<table>
<thead>
<tr>
<th>Clinical statusa</th>
<th>IFN-γ ELISPOT response status, no. of patients</th>
<th>Treated patients</th>
<th>Untreated patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Responderb</td>
<td>Nonresponderc</td>
<td>Responderb</td>
</tr>
<tr>
<td>Group 0</td>
<td>25</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>Group 1</td>
<td>7</td>
<td>1</td>
<td>...</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>11</td>
<td>26</td>
</tr>
</tbody>
</table>

a Group 0 consisted of *Trypanosoma cruzi*-seropositive individuals with normal findings on electrocardiographs and chest radiographs, and group 1 consisted of *T. cruzi*-seropositive patients with normal chest radiograph findings but abnormal findings on electrocardiographs, according to Kuschnir classification [24].

b IFN-γ ELISPOT responses greater than background levels at the initiation of the study.

c IFN-γ ELISPOT responses less than background levels at the initiation of the study.
Treatment Efficacy in Chagas Disease

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Figure 1. Effect of benznidazole treatment on Trypanosoma cruzi–specific T cell responses in chronic infection. Interferon (IFN)–γ–producing T cells specific for a parasite lysate per 1 × 10⁶ peripheral blood mononuclear cells were determined by enzyme-linked immunosorbent spot at baseline and 12 months after treatment with benznidazole. Each line represents an individual subject. *P = .041 for pretreatment differences between treated and untreated groups, as described in Methods; determined by Mann-Whitney U test.

Multiplex serodiagnostic assay. Serum specimens were screened for antibodies reactive to a panel of 14 recombinant T. cruzi proteins in a Luminex-based format, as previously described [26]. Serological responses to each individual T. cruzi protein were considered to have decreased during the study period if the mean fluorescence intensity decreased by ≥50% relative to that of the time 0 (pretreatment) sample assayed concurrently.

Statistical analysis. Comparisons on the frequencies of IFN-γ–producing T cells and on the percentages of CD4⁺ or CD8⁺ T cells expressing different phenotypic markers were performed using the Mann-Whitney U test of posttreatment and pretreatment differences between treated and untreated groups. The proportion of subjects for whom IFN-γ ELISPOT or B cell responses decreased over time in the treated and untreated groups was compared using the Fisher exact test. T cell responses at different time points were compared using the Friedman range test. A Spearman correlation test was applied to

Figure 2. Monitoring of interferon (IFN)–γ enzyme-linked immunosorbent spot (ELISPOT) responses in subjects with chronic Chagas disease who had positive ELISPOT responses at baseline. IFN-γ–producing T cells were measured at different time points after benznidazole treatment or enrollment (for untreated subjects). Plots represent the data for single subjects from a selected group. The IFN-γ–secreting T cell count significantly increased 2–6 months after treatment and decreased thereafter, as determined by Friedman range test (P = .012).
analyze the association between the frequency of IFN-γ–producing T cells and serological titers. Differences were considered to be statistically significant at \( P < .05 \).

**RESULTS**

**IFN-γ and IL-2 responses following treatment with benznidazole.** We monitored IFN-γ ELISPOT responses in 43 persons with chronic *T. cruzi* infection who were treated with benznidazole and a group of 32 untreated individuals observed over a 3–5-year period. Subjects were grouped according to pretreatment or baseline IFN-γ ELISPOT responses to an amastigote lysate into those with IFN-γ ELISPOT responses that were greater than background levels (“responder subjects”) and those with negative IFN-γ ELISPOT responses (“nonresponders”) (Table 1). There were no significant differences in the magnitude of the baseline IFN-γ ELISPOT responses between treated and untreated subjects for either responder and nonresponder subjects (\( P = .2 \)) or for responder subjects only (\( P = .10 \)). Within 12 months after treatment with a 30-day course of benznidazole, the frequency of IFN-γ–producing T cells specific for *T. cruzi* significantly decreased in the treated responder group, compared with the untreated group, who displayed relatively stable numbers of *T. cruzi*–responsive T cells (Figure 1).

To determine the earliest time point at which the decrease in *T. cruzi*–responsive T cells could be observed, IFN-γ responses were also measured 2 and 6 months after treatment in a subset of subjects. Surprisingly, a proportion of benznidazole-treated subjects (7 of 19 evaluated), but not of benznidazole-ununtreated subjects (0 of 7), exhibited an initial increase in IFN-γ responses before the decrease at 12 months (Figure 2). This same pattern of transient increase in T cell responses followed by its decay was also observed in 3 of 7 subjects who tested negative for *T. cruzi*–induced IFN-γ–producing cells before treatment (ie, nonresponder subjects PP19, PP21, and PP385) (Figure 3). The monitoring of IFN-γ production in a subset of subjects at multiple time points both before and after benznidazole treatment further corroborates the impact of treatment on parasite-specific T cell responses, demonstrating relatively stable responses before treatment followed by dramatic decreases 12–36 months after treatment in 3 of 4 subjects (subjects PP118, PP06, and PP277) (Figure 4). The majority of the IFN-γ–producing CD4+ T cells at 2 or 6 months after treatment were CD122lo, CCR7lo, and KLRG1lo, whereas a proportion of these CD4+IFN-γ+ cells express CD127 (Figure 5), consistent with an \( \text{T}_{\text{EM}} \) or \( \text{T}_{\text{E}} \) phenotype. Cumulatively, IFN-γ ELISPOT responses fell below the level of detection (47%) or decreased substantially (25%) in the majority of benznidazole-treated individuals but were unaltered in nontreated subjects (Table 2).

Benznidazole treatment did not alter the previously reported infrequent detection of IL-2–producing T cells responsive to *T. cruzi* in these subjects [21]. However, in individuals with IL-2–producing cells, the frequency of these cells changed in concert with IFN-γ+ T cell responses (Figure 4), demonstrating that the alteration in T cell responses generated by therapeutic treatment was not restricted to the production of a single cytokine. Interestingly, 3 subjects (subjects PP01, PP120, and PP179) who became negative for both IFN-γ and IL-2 re-
Monitor interferon (IFN)–γ and interleukin (IL)–2 enzyme-linked immunosorbent spot (ELISPOT) responses in subjects with chronic Chagas disease after treatment with benznidazole. IFN-γ–producing (solid line) and IL-2–producing (broken line) T cells were measured at different time points of follow-up in benznidazole-treated and untreated subjects. Time 0 indicates the assay point just prior to benznidazole treatment. Plo ts show representative data for single subjects whose IFN-γ ELISPOT responses became negative during follow-up (PP06), decreased >3 fold (PP118 and PP277), showed a rebound (PP01, PP120, and PP179), or remained stable (PP96, PP100 and RD31). The criteria for determining positive responses are defined in Methods.

Responsive by 12–24 months after treatment had a rebound in cells producing both cytokines by 36 months after treatment (Figure 4).

Taken together, these results show that benznidazole treatment induced substantial changes in T. cruzi–specific T cell responses in a significant proportion of benznidazole-treated subjects, suggestive of a decrease in antigen load and, possibly, parasite clearance.

Changes in serologic and clinical status after benznidazole treatment. Not surprisingly, given the relatively short follow-up period, the changes in the frequency of T. cruzi–specific IFN-γ–producing T cells correlated poorly with the titers of anti–T. cruzi antibodies, as measured using conventional [5, 7, 8] assays (data not shown); in only 6 of 43 treated subjects did the results of conventional serological tests become negative during the follow-up period, and in 5 of these 6 subjects, the level of IFN-γ–producing T cells also decreased to less than background levels. In addition, only 3 subjects exhibited progression in the assessment of disease severity during the follow-up period (2 in the benznidazole-treated group and 1 in the untreated group). However, in sharp contrast to conventional serologic tests, the changes in serologic status determined by a recently developed multiplex serodiagnostic assay [26] showed very strong correlation with both the treatment group and with changes in T cell responses after treatment (Table 3). Antibody titers to 14 recombinant T. cruzi proteins were essentially unchanged in untreated subjects (Figure 6A and 6E), with only 2 of 30 subjects observed for 12–36 months showing slight alterations over time. Alternatively, more than one-half of the treated subjects had a >50% decrease in reactivity to at least 1 of the possible 14 target proteins (Figure 6C, 6D, and 6F). Collectively, 33 of the 43 treated subjects had significant changes in either or both T cell and B cell responses consistent with a change in infection status (Table 3).

DISCUSSION

This study aimed to tackle the major limitation of treatment in the chronic phase of Chagas disease, the assessment of treatment efficacy, by measuring changes in parasite-specific T cell
responses. To our knowledge, this is the first report to describe the monitoring of *Trypanosoma cruzi*-specific T cell responses after benznidazole treatment of chronically infected adult subjects and the first to clearly associate alterations in T and B cell responses with proposed treatment efficacy. We found that the frequency of *T. cruzi*-specific IFN-γ–producing T cells decreased ≤1 year after benznidazole treatment in the majority of subjects and was less than the level of detection in nearly 50% of treated individuals. Similar changes in T cell responses were not evident either prior to treatment in the same individuals or in untreated subjects over the same observation time. In addition, changes in T cell responses were highly correlated with decreases in serological responses, as determined using a multiplex assay, but not as assessed by conventional serologic testing. Together
these data argue strongly that benznidazole treatment has a significant impact on chronic T. cruzi infection in humans.

The most straightforward interpretation of these results is that benznidazole treatment decreases parasite load, thus diminishing the antigen necessary to activate T. cruzi–specific T cells and B cells. It is also reasonable to hypothesize that the treatment actually cures T. cruzi infection in a substantial number of treated subjects. Similar positive effects of benznidazole treatment have been well documented in animal models and in early/acute infections [1–4, 27, 28], but it has been argued that treatment is ineffective in chronically infected humans [29, 30]. However, the conclusion that drug treatment in chronic Chagas disease is ineffective is based largely on the lack of effective means to assess treatment efficacy rather than actual data demonstrating the lack of efficacy. Indeed, a number of clinical studies with 8–10-year follow-up periods [5, 7, 8] have shown that drug treatment during the chronic phase of T. cruzi infection significantly decreases the progression of clinical disease. Although these studies are criticized because they are not randomized and/or placebo controlled, they nonetheless are highly suggestive of cure of the infection in a substantial number of drug treated subjects—something that is rarely observed in the absence of drug treatment.

Unfortunately, there is no ethical way to determine with

Table 2. Cumulative Decreases in Interferon (IFN)–γ T Cell Responses after Treatment with Benznidazole

<table>
<thead>
<tr>
<th>Time after enrollment</th>
<th>Negative IFN-γ ELISPOT results</th>
<th>&gt;3-Fold decrease</th>
<th>Total</th>
<th>Proportion of untreated patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Months</td>
<td>5/26</td>
<td>4/26</td>
<td>9/26</td>
<td>0/13</td>
</tr>
<tr>
<td>24 Months</td>
<td>8/32</td>
<td>7/32</td>
<td>15/32</td>
<td>1/23</td>
</tr>
<tr>
<td>36 Months</td>
<td>15/32</td>
<td>8/32</td>
<td>23/32</td>
<td>1/26</td>
</tr>
</tbody>
</table>

NOTE. ELISPOT, enzyme-linked immunosorbent spot.

a IFN-γ ELISPOT responses were less than detectable levels at ≥1 point after benznidazole treatment.

b IFN-γ ELISPOT responses decreased by >3-fold relative to pretreatment levels.

c $P = .04$, compared with untreated patients, by the Fisher exact test.

d $P < .001$, compared with untreated patients, by the Fisher exact test.
Figure 6. Multiplex analysis of anti-\textit{Trypanosoma cruzi} antibodies before and after treatment. Serum specimens obtained at the indicated time points were screened using a bead array-based multiplex serologic assay against 14 recombinant \textit{T. cruzi} proteins (antigens 1–14), as well as against a negative control protein (green fluorescent protein) and an \textit{T. cruzi} amastigote lysate, as previously described in detail [26]. Subjects who were not treated (A) or treated (B) with benznidazole (at time $t_0$) did not exhibit a consistent change in antibody levels over 36 and 31 months, respectively. Different patterns of alterations in antibody responses was observed in treated subjects, including changes in antibodies specific for all (C) or just some (D) of the \textit{T. cruzi} proteins. Subject PP118 had a stable pattern of antibodies for 12 months before treatment (E) but experienced a steady decrease in responses to most proteins apparent within 12 months following treatment (F). *No data (ie, insufficient numbers of beads recovered for accurate measurement).

100\% certainty the efficacy of drug treatment in chronic human \textit{T. cruzi} infection. We have used immunosuppression with cyclophosphamide to demonstrate that benznidazole can achieve parasitological cure of mice with chronic \textit{T. cruzi} infection [31]. However, because direct detection of \textit{T. cruzi} or it productions or constituents (eg, DNA and proteins) is unreliable in chronically infected hosts, including humans [32–34], these same measures are not informative with respect to the determining of the effectiveness of any treatment. Thus, assessment of treatment efficacy has generally relied on measurement of disease progression and conventional serological responses—both of which require up to a decade after treatment to effectively evaluate. Indeed, in this study, we could not correlate changes in conventional serology or clinical status with the other immunological changes noted at earlier time points after treatment. However, previous studies by our group using the identical treatment protocol and similar patient populations found a significant decrease in progression to disease among benznidazole-treated subjects, compared with untreated subjects, 8–10 years after treatment [5, 7].

In contrast to the results herein, a recent study from Fernandez et al [35] reported a very high failure rate of treatment based on polymerase chain reaction–based detection of \textit{T. cruzi}, both before and after treatment. However, all subjects became hemaculture negative after treatment and demonstrated consistently decreasing serological titers during the 3-year follow-up period, both indicative of a decreasing parasite load in these subjects rather than complete treatment failure.

We attribute the decline in \textit{T. cruzi} -specific T cell responses after benznidazole treatment to decreased parasite antigen...
needed to drive antigen-dependent effector T cells. Interestingly, the reduction in T. cruzi–specific T cells was often preceded by an increase in \( T_{ef} / T_{EM} \) IFN-\( \gamma \)–producing cells early after treatment, perhaps as a result of the release of parasite antigens as a consequence of drug-induced parasite death. Although this response was not documented in all subjects, this may have been the result of infrequent sampling.

A subset of subjects also had a rebound in parasite-specific T cell responses very late (>24 months) after treatment. Unfortunately, the frequency of these cells was not sufficiently high to allow for the collection of reliable phenotyping data. However, this rebound was associated with decreasing serological titers by the multiplex assay, and thus, it seems likely these parasite-specific T cells are maintained in the absence of antigen—a characteristic of \( T_{CM} \) as shown in mice that are cured of T. cruzi infection [31]. Ultimately, a metric such as a high percentage of \( T_{CM} \) phenotype cells among the parasite-specific T cell population (when they can be measured) may be the best surrogate for assessing treatment efficacy in individual patients.

One of the drawbacks to the assessment of T. cruzi–specific T cell responses as an indicator of treatment efficacy in chronically infected subjects is the fact that these responses were less than the level of detection in nearly one-fourth of the individuals. This result is consistent with previous studies showing low and often undetectable parasite-specific T cell responses [21, 25] along with a differentiated status of the overall CD8\(^+\) T cell pool [22] in individuals with decades-long infections with T. cruzi, indicating a more general dysfunction of the immune system during chronic infection as a consequence of persistent activation [13, 14, 18, 36].

On the basis of the substantial changes in immunological responses and on the arrest in disease progression reported in previous studies [5, 7], we conclude that the protocol for benznidazole treatment used in these subjects is effective in altering the course of infection and likely achieves parasitological cure in up to three-fourths of subjects with decades-long infections. Importantly, this estimate is in line with the 76%–83% reduction in the relative risk of progression in benznidazole-treated subjects reported in several studies [6, 37, 38], including those using a similar patient group and an identical treatment protocol to that used in the current study [5, 7].

Nevertheless, benznidazole treatment is not uniformly effective, because immunological changes are not observed in all treated individuals, and progression to more-severe disease continues after treatment in a small proportion of cases [5, 7]. This result is not surprising—various T. cruzi isolates are differentially susceptible to the actions of benznidazole, and even among inbred mice infected with the identical dose and strain of T. cruzi, not all animals are cured when treated with a shorter course (<40 days) of benznidazole [31].

It will be both important and interesting to continue to observe the subjects in this study to determine whether the observed early changes in immunological parameters we report here correlate with long-term disease progression. However, in the interim, we suggest that changes in T. cruzi–specific T cell responses and in antibody responses to individual parasite proteins can be used as early and effective predictors of the effectiveness of drug treatment in human Chagas disease. Furthermore, these results strongly challenge the contention that drug therapy is ineffective in chronic Chagas disease and provide additional support for the wider use of existing drugs, despite their imperfections, to treat the millions of individuals who are infected and who are likely to develop clinical disease [39, 40]. As importantly, we believe the tools developed and assessed in this study will help to remove a major barrier to the development and testing of improved drugs for the treatment of T. cruzi infection.

**Acknowledgments**

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**References**


