

## Implication of Transforming Growth Factor- $\beta$ 1 in Chagas Disease Myocardiopathy

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Cardiac dysfunction with progressive fibrosis is a hallmark of Chagas disease. To evaluate the involvement of transforming growth factor (TGF)- $\beta$ 1 in this disease, TGF- $\beta$ 1 levels in patients were measured at 3 stages: asymptomatic indeterminate (IND), cardiac with no or slight heart dysfunction (Card 1), and cardiac with moderate or severe heart dysfunction (Card 2). All patients had significantly higher circulating levels of TGF- $\beta$ 1 than did healthy persons, and 27% of patients in the Card 1 group had higher TGF- $\beta$ 1 levels than did patients in the IND group. Immunohistochemical analysis of cardiac biopsy specimens showed strong fibronectin staining in the extracellular matrix and staining for phosphorylated Smad 2 (activation of the TGF- $\beta$ 1 signaling pathway) in cell nuclei. The higher levels of latent TGF- $\beta$ 1 observed in patients with myocardiopathy, together with intracellular activation of the TGF- $\beta$ 1 pathway and tissue fibrosis, suggest that TGF- $\beta$ 1 plays an important role in Chagas disease. TGF- $\beta$ 1 may represent a new target for preventive and curative treatments of Chagas disease.

Cardiac damage and dysfunction are prominent features in patients with chronic Chagas disease, which is caused by infection with the protozoan parasite *Trypanosoma cruzi* and affects ~16–18 million individuals in South and Central America [1]. Each year, 45,000 patients with Chagas disease die in Latin America from the cardiac and digestive complications. The disease is characterized by the occurrence of a short acute phase followed by a long-lasting chronic phase, during which clinical symptoms may occur in ~30% of the infected persons [2]; however, 70% of seropositive patients remain asymptomatic during the so-called indeterminate (IND) stage of chronic Chagas disease.

The mechanisms leading to the development of Chagas dis-

ease are not yet well understood [3], but there is consensus regarding the important role of the host immune system. All patients are characterized by serological or parasitological positivity for *T. cruzi*. Patients with the chronic cardiac form of Chagas disease (Card) differ from patients with the asymptomatic IND form in that they have important alterations in the electrical conduction system and/or ventricular arrhythmias seen on abnormal electrocardiograms (ECGs). They also gradually develop congestive heart insufficiency, associated or not with cardiomegaly, seen on abnormal chest radiograms or echocardiograms (ECOs) [2]. In those patients with ongoing myocardiopathy, mononuclear infiltration is associated with the presence of parasite antigens. Microvascular alterations, ventricular remodeling, and fibrosis are also observed [4].

Transforming growth factor (TGF)- $\beta$ 1 plays a central role in fibrosis, contributing both to the influx and activation of inflammatory cells and to the activation of fibroblasts for extracellular matrix (ECM) production [5, 6]. It regulates a concert of actions, inducing the expression of several matrix components (e.g., fibronectin, collagen, vitronectin, proteoglycans, and thrombospondin), decreasing secretion of matrix-degrading proteases, and increasing the synthesis of protease inhibitors [7, 8]. Because TGF- $\beta$ 1 is abundantly distributed in the pericellular environment of many tissues, its activity is subjected to strict control [9]. TGF- $\beta$ 1 is secreted and stored at the cell surface and in the ECM mostly under a latent form. Two major systems contribute to sustain the physiological latency of TGF- $\beta$ 1: binding to the plasma protease inhibitor  $\alpha$ 2-macroglobulin (A2M) and complex formation with the latency-associated

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protein (LAP), and further association of the TGF- $\beta$ 1-LAP complex with the ECM-associated latent TGF- $\beta$ 1 binding protein. Binding of active TGF- $\beta$ 1 to its high-affinity type II receptor results in the activation of type I receptors and further phosphorylation of the Smad 2 and Smad 3 signaling proteins [10]. Their association with the common mediator Smad 4 leads to the translocation of this complex to the nucleus, where it participates in the regulation of the transcription of target genes, such as those encoding fibronectin and other ECM components [11].

A study of the expression and involvement of TGF- $\beta$ 1 in the development of chagasic myocardiopathy is lacking, regardless of the outstanding fibrosis characteristic of this disease. Increased biologically active TGF- $\beta$ 1 production was observed during the acute phase of experimental mouse infection by *T. cruzi* [12]. Activation of the TGF- $\beta$ 1 signaling pathway appears to be absolutely required for cell invasion by *T. cruzi* [13]. However, most of studies that have dealt with TGF- $\beta$ 1 and *T. cruzi* infection have focused on its role as an immune modulator [14, 15].

In the present work, we aimed to measure serum levels of TGF- $\beta$ 1 in *T. cruzi*-infected patients with chronic Chagas disease who presented with different clinical forms and to correlate TGF- $\beta$ 1 expression with progressive fibrotic lesions in the heart (assessed by a decreased ventricular ejection fraction) and by fibronectin immunostaining in heart biopsy specimens. Our results show that patients with Chagas disease have ~10–20-fold higher levels of serum TGF- $\beta$ 1 than do uninfected healthy individuals. Heart cells from patients with moderate or severe heart dysfunction react strongly with antibodies against phosphorylated Smad 2 (PS2) [9–11], a marker of activation of the TGF- $\beta$ 1 signaling pathway, and against fibronectin, a marker of fibrosis.

## Patients, Materials, and Methods

**Patients with chronic Chagas disease.** Blood samples were obtained from 73 patients during clinical survey at the Instituto de Pesquisa Clínica Evandro Chagas, Rio de Janeiro. These patients (35 men and 38 women; age range, 22–74 years) had confirmed positive serologic test results for *T. cruzi* infection. They were regularly monitored by clinical examination and ECG and ECO recording, to check for electrical abnormalities and for their ventricular ejection fraction (VEF). Blood donation was voluntary. The individuals were classified into 3 groups according to the severity of Chagas disease, as assessed by clinical examination and ECG and ECO results. The first group, IND ( $n = 22$ ), corresponded to functional class I, as defined by the New York Heart Association (NYHA; 2001 Practice Guidelines are available at [http://www.hfsa.org/pdf/lvsd\\_heart\\_failure.pdf](http://www.hfsa.org/pdf/lvsd_heart_failure.pdf)). The second group, Card 1 ( $n = 34$ ), included patients with cardiac Chagas disease who had ECG alterations and slight or no heart dysfunction, corresponding to NYHA functional class II. The third group, Card 2 ( $n = 17$ ), included patients with cardiac Chagas disease who had ECG and/or ECO alterations and moderate or severe heart dysfunction, corresponding to NYHA functional classes III–IV. Reference healthy adult (HA) blood samples were taken from donors at the blood bank of the University Hospital

of the Federal University of Rio de Janeiro ( $n = 12$ ; 5 men and 7 women).

**Biopsy specimens.** Heart fragments were obtained from 6 patients with Chagas disease, 2 who died from heart insufficiency and 4 who underwent heart transplantation (heart explanted from the receivers: 2 cases of NYHA class III, a Bolivian man 53 years old and a Brazilian woman 39 years old, and 2 cases of NYHA class IV, 2 Brazilian men 49 and 56 years old). As uninfected controls, we used heart fragments from 2 patients with noninfectious dilated cardiopathy who also underwent heart transplantation (both NYHA class III, 1 man 66 years old, presenting with ischemic dilated cardiopathy with a VEF of 38%, and 1 woman 19 years old, presenting with idiopathic dilated cardiopathy), as well as from 1 young patient who presented with normal heart function but was given a valve prosthesis.

**TGF- $\beta$ 1 measurement.** Measurement was performed using a TGF- $\beta$ 1-specific commercial ELISA kit (TGF- $\beta$ 1 Emax; Promega), according to the manufacturer's instructions. All samples were assayed after acidic pH activation of latent TGF- $\beta$ 1; some selected chagasic samples were assayed also without activation, to calculate the ratio between active and latent TGF- $\beta$ 1.

**Anti-*T. cruzi* IgG immune reactivity.** ELISA reagents were obtained from Sigma Chemical. Serum samples were diluted 1:500 and assayed on plates coated with 0.5  $\mu$ g/mL *T. cruzi* soluble antigen, as described elsewhere [16]. Results were expressed as an index of reactivity.

**A2M measurement.** A2M was quantitated by ELISA, as described elsewhere [16].

**Fibronectin and PS2 detection in heart cryosections.** Heart fragments from healthy patients, from hearts of patients with noninfectious myocardiopathy, or from patients with Chagas disease were obtained and cut in a cryostat. The histologic analysis and immune labeling of *T. cruzi* antigens and of inflammatory infiltrates of these patients have already been studied and described elsewhere [17–19]. Sections were fixed in paraformaldehyde, incubated with 1% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase, further washed, and saturated with 5% normal goat serum before an overnight incubation with rabbit IgG anti-fibronectin (Sigma) or anti-PS2. The anti-PS2 antibody was raised in rabbits against the peptide KKKSSpMSP (where Sp stands for phosphorylated serine residues) and was kindly given to us by P. ten Dijke (The Netherlands Cancer Institute, Amsterdam). Its specificity was assessed by Western blotting and immunochemical methods [20]. A biotin-avidin-peroxidase system (Dako) was used for immune detection.

**Statistical analysis.** Nonparametric methods were used, because most of the data did not have normal distribution. The Kruskal-Wallis and Mann-Whitney *U* tests were applied to medians from the groups under study. Frequency distribution differences were assessed by the Kolmogorov-Smirnov test. Spearman's test was applied to ascertain correlation between the different variables. Statistical significance was defined as  $P < .05$ .

## Results

Our first goal was to measure circulating TGF- $\beta$ 1 levels in the serum of patients with various stages of Chagas disease. Measurement of VEF confirmed the absence of heart dys-

**Table 1.** Values of ventricular ejection fraction (VEF), transforming growth factor (TGF)-β1, α2-macroglobulin (A2M), and anti-*Trypanosoma cruzi* (anti-*Tc*) IgG reactivity during the progression of chagasic myocardiopathy.

Subject group	Age, years	VEF, %	Anti- <i>Tc</i> IgG, IR	TGF-β1, ng/mL	A2M, mg/mL
HA	42.5 [43.9 ± 9.2] (12)	ND	0.5 [0.5 ± 0.1] (12)	0.44 [2.2 ± 3.2] (12)	11.5 [11.3 ± 3] (12)
IND	50.5 [48.4 ± 10] (22)	68.5 [69.5 ± 7.4] (22)	1.9 [2.1 ± 0.6] (12) <sup>a</sup>	5.3 [9.9 ± 11.6] (22) <sup>a</sup>	13 [13.4 ± 5.5] (12)
Card 1	50 [50.5 ± 9] (34)	62 [62.7 ± 9.5] (34)	3.1 [3.2 ± 0.9] (14) <sup>a</sup>	21.4 [30.4 ± 31.2] (34) <sup>a,b</sup>	12.5 [11.9 ± 4.6] (14)
Card 2	49 [48.8 ± 11] (17)	40 [38.3 ± 8.2] (15) <sup>b</sup>	3.8 [3.6 ± 1.6] (8) <sup>a</sup>	17.8 [41.4 ± 60.3] (17) <sup>a</sup>	13.2 [13.4 ± 6.8] (9)

NOTE. Data are median [mean ± SD] (no. of subjects). Card 1, electrocardiogram (ECG) alterations, heart dysfunction 0 or 1; Card 2, ECG alterations, heart dysfunction 2 or 3; HA, healthy adult; IND, chronic indeterminate, asymptomatic; IR, index of reactivity.

<sup>a</sup> *P* < .05, vs. the HA group.

<sup>b</sup> *P* < .05, vs. the IND group.

function in the IND and Card 1 groups, whereas patients in the Card 2 group showed moderate or severe heart insufficiency, as indicated by a VEF value of 18%–48% (table 1). The immune response to *T. cruzi* antigens augments with the progression of the disease (table 1) and inversely correlates with the VEF values (*r* = -0.47; *P* = .006; *n* = 32; data not shown).

As shown in table 1, the 3 different groups of patients with chronic Chagas disease had significantly higher circulating TGF-β1 levels than the control group. The median seric TGF-β1 concentration was highest in the Card 1 group (21.4 ng/mL) and was significantly higher than the levels found in patients from the IND group. Serum TGF-β1 concentrations in the Card 2 group were more dispersed. A study of frequency distribution performed on these values (table 2) clearly showed an increased percentage of patients with abnormally high serum TGF-β1 levels in all 3 groups; 36% of patients from the IND group, 61% from the Card 1 group, and 71% from the Card 2 group presented with TGF-β1 levels >10 ng/mL, which was considered to be a cutoff value, whereas 100% of the healthy donors had TGF-β1 values under this threshold value (table 2). In the Card 2 group, we found 2 patients with TGF-β1 levels >200 ng/mL (214.5 and 230 ng/mL), corresponding to those who had VEF values of 40% and 38%. However, the Spearman's test did not indicate any direct negative or positive correlation between TGF-β1 levels and VEF values. Indeed, the 5 patients in the Card 2 group with normal TGF-β1 levels (table 2) had VEF values of 40%, 38%, 36%, 27%, and 18%, respectively. Because the immune reactivity for *T. cruzi* is an indirect indicator of parasitic load in the tissues [21] and because cells from infected mice appear to release active TGF-β1 in culture [12], we checked whether at least part of the total latent TGF-β1 was already in the active form. The samples were assayed with or without the acidic pH activation step required for immune detection of active TGF-β1 by ELISA, and the results indicated that TGF-β1 was totally under a latent form in all serum samples (data not shown).

Because A2M associates with TGF-β1 in serum to form a latent complex and because we have already shown that asymptomatic children acutely infected with *T. cruzi* have significantly higher A2M levels than do symptomatic children [16], we measured A2M levels in the serum of patients with Chagas disease (table 1). However, A2M levels were similar in all groups tested. The finding of a significant frequency of patients with cardiac

Chagas disease with high TGF-β1 serum levels suggested that this cytokine could be involved in the genesis of the fibrotic events that lead to ventricular remodeling and heart dysfunction [3, 22]. We then analyzed heart biopsy specimens from patients with cardiac Chagas disease by immunohistochemical methods for the presence of fibronectin and of PS2, markers of fibrosis and active TGF-β1 signaling, respectively. Strong nuclear PS2 staining and pericellular fibronectin staining were observed on the sections of cardiac tissue from patients with myocardiopathic Chagas disease (figure 1C and 1F). Low background staining was visible when the first antibodies were omitted (figure 1A, 1B, and 1C, insets). Of interest, both fibronectin and PS2 stainings appeared to be specific to heart biopsy specimens from patients with Chagas disease, because the sections of heart biopsy specimens from a healthy individual (figure 1A and 1D) or from a patient with noninfective myocardiopathy (figure 1B and 1E) were much less intensely stained. Similar observations were made in a total of 5 different biopsy specimens.

**Discussion**

Here, we show for the first time that patients with Chagas disease have higher circulating TGF-β1 levels than healthy volunteers. A significantly greater proportion of patients in the Card 1 group than patients in the IND group had TGF-β1

**Table 2.** Frequency distribution of transforming growth factor (TGF)-β1 levels during the progression of chagasic myocardiopathy.

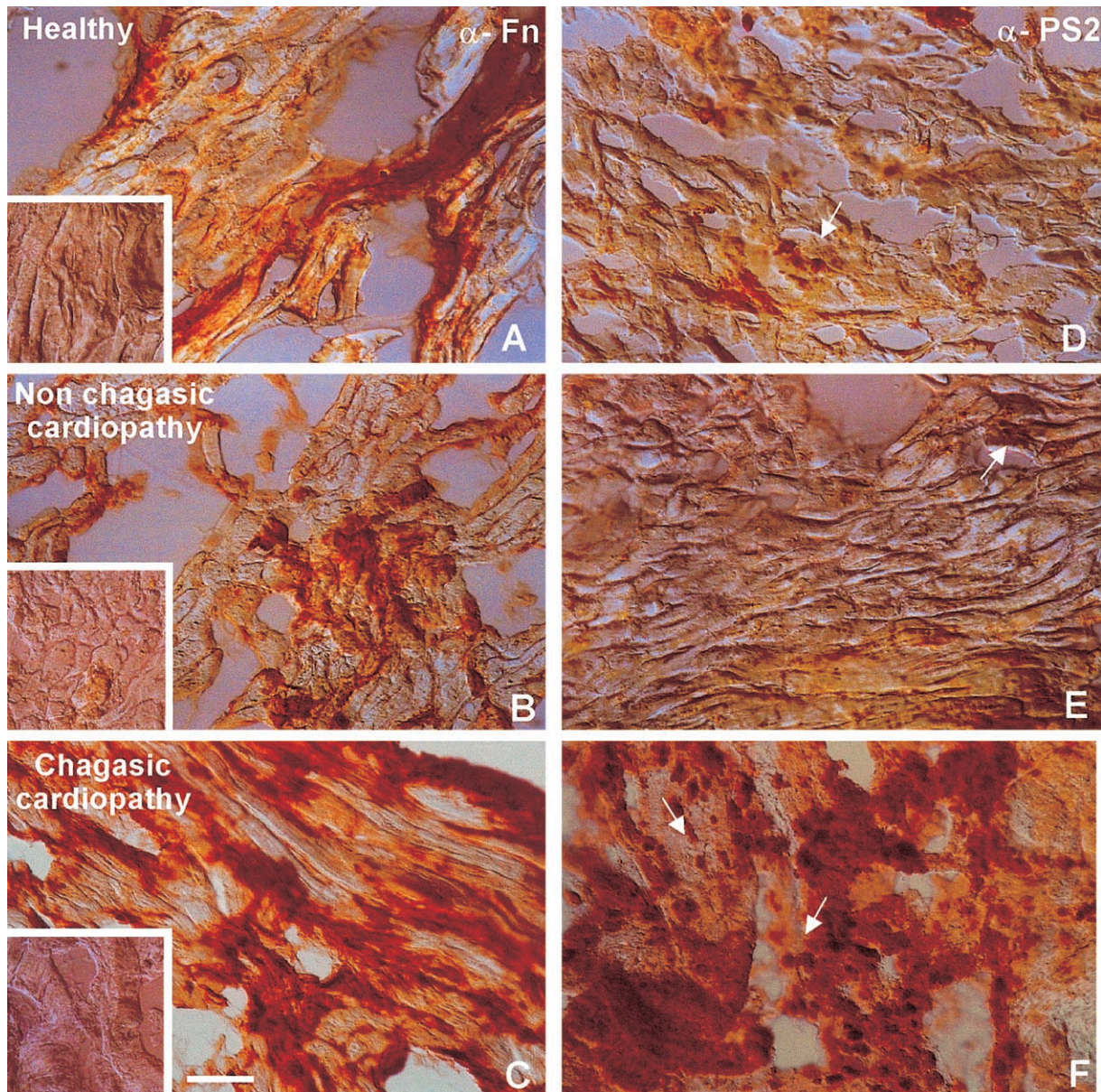
TGF-β1 level, ng/mL	HA	IND	Card 1 <sup>a,b</sup>	Card 2 <sup>a</sup>
<10	12/12 (100)	14/22 (64)	13/34 (39)	5/17 (29)
10–20	0	2/22 (9)	4/34 (12)	4/17 (24)
21–30	0	5/22 (23)	3/34 (9)	3/17 (18)
31–40	0	1/22 (5)	5/34 (15)	1/17 (6)
41–50	0	0	3/34 (9)	2/17 (12)
51–100	0	0	4/34 (12)	0
101–200	0	0	2/34 (6)	0
≥201	0	0	0	2/17 (12)

NOTE. Data are no. of samples in each range of frequency/total no. of samples in the group (%). Card 1, electrocardiogram (ECG) alterations, heart dysfunction 0 or 1; Card 2, ECG alterations, heart dysfunction 2 or 3; HA, healthy adults; IND, chronic indeterminate, asymptomatic.

<sup>a</sup> *P* < .05, vs. the HA group.

<sup>b</sup> *P* < .05, vs. the IND group.





**Figure 1.** Markedly elevated expression of anti ( $\alpha$ )-phosphorylated Smad 2 (PS2) and  $\alpha$ -fibronectin (Fn) in heart biopsies from patients with Chagas disease with myocardiopathy. Sections from heart biopsy specimens were stained for Fn (A–C) and PS2 (D–F), as described in Patients, Materials, and Methods. Immunoreactivity was visualized as brown color. Fragments of heart biopsy specimens from a healthy donor (A and D), from a patient without Chagas disease with idiopathic dilated cardiopathy (B and E), or from a patient with chagasic myocardiopathy (C and F) were analyzed. Specificity of the staining is attested by the negative controls (insets in panels A–C), in which serial sections from the biopsy specimens examined in panels A–F were incubated in the absence of the primary antibody. Representative results from 1 of 5 similarly analyzed heart biopsy specimens are shown. Arrows, PS2-stained nuclei of heart muscle. Bar, 500  $\mu$ m. All pictures were taken at 400 $\times$  magnification.

levels >10 ng/mL. TGF- $\beta$ 1 is under a latent form in serum, probably associated with A2M [23]. TGF- $\beta$ 1 could then be a useful prognostic marker of disease progression, and a prospective study on a large population of patients in the IND form of disease would indicate whether the increase in TGF- $\beta$ 1 levels could predict which patient in the IND stage of disease will progress to the Card 1 stage of disease.

The presence of PS2 in heart-cell nuclei of patients with Chagas disease is a very strong evidence for the activation of the TGF- $\beta$ 1 pathway in these cells. A similar nuclear labeling was observed in bronchial epithelial, lung alveolar, and infiltrating cells in a mouse model of airway inflammation associated with TGF- $\beta$ 1 activation [20]. The presence of PS2 may result from higher levels of active TGF- $\beta$ 1 or from decreased levels of pro-

teins that down-regulate TGF- $\beta$ 1 signaling, such as decorin [24], which prevents binding of TGF- $\beta$ 1 to its serine/threonine kinase receptors. Increased expression of TGF- $\beta$ 1 mRNA and protein in myocardium bordering the infarct region have been associated with cardiac wound-healing response [24]. Because, in patients with Chagas disease, there is microthrombus formation, micromyocardial infarction, and inflammatory reaction [22, 25], the enhancement of TGF- $\beta$ 1/Smad signaling might reflect a secondary reaction to the remodeling of the infarct scars after completion of wound healing per se. However, it is also possible that the enhanced TGF- $\beta$ 1 pathway activation that we observed could be directly involved in the intense synthesis and secretion of ECM molecules (including fibronectin) occurring in the fibrotic process. Whether activation of TGF- $\beta$ 1 results from myocardial infarction or whether myocardial infarction results from TGF- $\beta$ 1-induced fibrosis is still an open question. A previous immunohistochemical analysis performed on 19 patients with moderate-to-severe heart dysfunctions who died from Chagas disease cardiopathy revealed positive cardiac staining for platelet-derived growth factor (PDGF)-B, PDGF-A, and TGF- $\beta$ 1 [26]. Those results corroborate our present findings. It is possible that the early increase in TGF- $\beta$ 1 levels observed in patients at the Card 1 stage of disease triggers later effects and/or contributes to the establishment of a fibrotic process in concert with other growth factors. The contrasting patterns of fibronectin and PS2 detection in the hearts of patients with infectious (figure 1F) and idiopathic (figure 1D) Chagas disease supports a role of parasite antigens in the development of chagasic fibrosis, because the pattern of fibrosis is distinct under both conditions [22], similar to what was described in hepatic fibrosis induced by *Schistosoma mansoni* antigens [27]. Circulating CD8<sup>+</sup> T cells are commonly detected in chagasic myocardiopathy and in heart rejection reaction but are not common in idiopathic dilated cardiopathy [18].

The high TGF- $\beta$ 1 serum levels that we observed in the present study could also derive from a direct stimulation of TGF- $\beta$ 1-producing cells by the parasite. The facts that (1) anti-*T. cruzi* IgG immune reactivity can be reversed by trypanocide chemotherapy [21], whereas lymphocyte activation remains sustained [28], (2) inflammatory lesions in the myocardium of patients with Chagas disease are directly correlated with the presence of *T. cruzi* [4, 18], and (3) a high number of heart cells contain nuclear PS2 suggest that antigens from a parasite stock in the heart or in another site in the infected patient may sustain an activated TGF- $\beta$ 1 signaling pathway that leads to increased fibrosis. Because it has been shown that TGF- $\beta$ 1 facilitates parasite invasion by deactivating macrophage trypanocidal mechanism [12], the increased levels of TGF- $\beta$ 1 could contribute to sustain the parasitic load in patients with chronic disease. This would be in agreement with the progressive, increasing anti-*T. cruzi* immunoreactivity that we observed as a function of disease progression (table 1; index of reactivity for anti-*T. cruzi* IgG: HA, 0.5; IND, 1.9; Card 1, 3.1; and Card 2, 3.8).

Taken together, our results suggest that monitoring TGF- $\beta$ 1 levels and activity could be useful in the follow-up of patients with Chagas disease. It could help to determine the moment of rupture of the immune equilibrium that starts in fact the disease. It could also help to select the patients with chronic disease, who should receive specific chemotherapy, to prevent further development of chagasic myocardiopathy. The identification of TGF- $\beta$ 1 as an important actor of chagasic myocardiopathy may also pave the way to the development of new preventive or curative treatments for this complication of Chagas disease. Anti-TGF- $\beta$ 1 antibodies, which have been successfully used to treat experimental fibrotic nephropathy [29], are candidate molecules for the treatment of chagasic cardiac fibrosis.

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