Cellular Immune Response From Chagasic Patients to CRA or FRA Recombinant Antigens of *Trypanosoma cruzi*

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We propose to analyze the relation between the cellular immune response of Chagas' disease patients after in vitro stimulation of peripheral blood mononuclear cells (PBMC) with recombinant antigens cytoplasmatic repetitive antigen (CRA) or flagellar repetitive antigen (FRA) of *T. cruzi* and the chronic clinical forms of disease. Cells were stimulated using phytohemagglutinin, CRA, FRA, or a soluble antigen of Epimastigota (Ag-Epi) for 24 hr, 72 hr, or 6 days. The proliferation of cells was evaluated after 6 days of culture by quantification of incorporated ³H-thymidine. Cytokines were measured in the supernatants obtained after 24 hr (tumor necrosis factor [TNF]- α and interleukin [IL]-4), 72 hr (IL-10), and 6 days (interferon [IFN]- γ) using enzyme-linked immunosorbent assay (ELISA). Cells of the Chagas patients stimulated with the recombinant antigens exhibited higher proliferation responses

compared with that of non-Chagas (NC) individuals. However, when proliferation was compared between patients with the cardiac form (CF) or indeterminate form (IF), it was not possible to establish a difference in the response. So far as the cytokines secreted in the culture supernatants after stimulation in vitro with *T. cruzi* antigens were concerned, the results showed that CRA, as well as Epi-Ag, were able to stimulate the production of TNF- α and IFN- γ in Chagas patients as compared with NC individuals. However, the cytokine levels after stimulation with the *T. cruzi* antigens were not different between the patients with CF and IF. CRA was capable of inducing a T helper type 1 (Th1) immune response, with elevated production of TNF- α and IFN- γ in Chagas patients that are carriers of CF and IF clinical forms. J. Clin. Lab. Anal. 22:91–98, 2008. © 2008 Wiley-Liss, Inc.

Key words: cytokines; recombinant antigens; Chagas' disease

BACKGROUND

Chagas' disease is considered an important public health problem, as the global prevalence of this human infection is estimated at around 16–18 million cases and approximately 120 million people are under risk of infection (1). It is estimated that around 25–30% of the individuals infected by *Trypanosoma cruzi* will develop irreversible clinical forms of the disease, causing considerable mortality and morbidity rates in populations of endemic countries (2).

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The pathology of the lesions that lead to severe forms of the disease is still unknown (3,4). With respect to the initial mechanisms involved in the development of Chagas' disease cardiomyopathy, some works suggest the participation of autoimmune mechanisms (4–6). However, other authors recognize the existence of a specific immune response to the antigens of the parasite that could contribute directly to the evolution of the disease (7–12).

Differences in the intensity of the proliferative cellular response between the asymptomatic form and the symptomatic forms have been reported by various authors. Cetron et al. (13) identified a greater intensity of cell proliferation in peripheral blood mononuclear cells (PBMC) of individual carriers of the indeterminate clinical form (IF) when compared to carrier patients of the cardiac form (CF) and digestive clinical forms after in vitro stimulation with trypomastigote lysate of *T. cruzi*. Inverse results were produced when the cell stimulation was performed with antigens obtained from epimastigote forms of the parasite (14). Differences in cellular reactivity were reported in individual carriers of severe CF to flagellar (69%) and cytosolic (63%) fractions of *T. cruzi* when compared to the results of asymptomatic individuals (38% and 27%, respectively) (15). However, other studies found no differences in the proliferative cell response between asymptomatic patients and these carriers of symptomatic forms after in vitro stimulation of PBMC with lysates from different strains and clones of epimastigote forms of *T. cruzi* (16); purified epimastigote fractions (17) and paraflagellar rod proteins (PFR) (18).

Complex *T. cruzi* antigens have been used to investigate patterns of cytokine secretions in PBMC of Chagas' disease patients (8,9,19). These studies demonstrated that the interleukin (IL)-10 is the cytokine secreted by IF patients, suggesting that this cytokine could be associated with the protection of the host against the development of chronic symptomatic forms. The CF patients, however, have high levels of interferon (IFN)- γ , being correlated with the level of severity of cardiac injury (9).

Few studies have been performed with purified antigens of the parasite. PBMC from chagasic individuals after in vitro stimulation with PFR secreted high levels of IFN- γ and tumor necrosis factor (TNF)- α . However, no difference was found between CF and IF individuals (18). The predominant response of IFN- γ was reported in cardiomyopathy patients when PBMCs were stimulated with trans-sialidase (20), with a recombinant *T. cruzi* B13 protein (21) or with purified subfractions of trypomastigote antigens (22). Another study, also showed the IFN- γ production after stimulation with cruzipain (23).

The use of molecularly defined sequences of the parasite is a requisite not just for the development of serological blood tests and screening in blood banks, but also for the identification of important molecules in parasite-host interaction and to understand processes related to the immunopathology of Chagas' disease (24,25).

Two recombinant antigens (Rec-Ags), cytoplasmatic repetitive antigen (CRA) and flagellar repetitive antigen (FRA), already studied in murine model (26–29), have been successfully used in the immunodiagnosis of Chagas' disease (30–33). These antigens were also used to evaluate the cure for Chagas' disease in patients from Minas Gerais (Brazil) treated in the acute phase of the infection (34). In a pilot study performed through our group, CRA and FRA were capable of inducing the production of cytokines in the PBMC of Chagas individuals (Pereira et al., unpublished results). FRA stimulated PBMC in CF patients to produce IFN- γ and TNF- α and, CRA stimulated the production of IL-10 by individual carriers of the IF form of the disease, suggesting that these antigens could identify distinct profiles of cytokines between individual carriers of the different clinical forms of the disease.

Considering the importance of the immune response against the antigens of the parasite, evidenced by the production of specific antibodies against CRA or FRA by Chagas patients (35) and by the potential prognostic value of Rec-Ags (Pereira et al., unpublished results), the present study evaluated the specific immune cellular response to these antigens by PBMCs of CF and IF Chagas individuals aiming to use prognostic markers of the evolution of severe clinical forms of Chagas' disease.

METHODS

T. cruzi Antigens

The CRA and FRA antigens were prepared in the Bio-Manguinhos Department of Reactives for Diagnostics and obtained as previously published by Krieger et al. (31). The soluble epimastigote antigen (Epi-Ag) from the Y strain of *T. cruzi* was obtained according to Pereira et al. (36).

Study Population

Patients carrying forms of chronic Chagas' disease were selected in the Chagas' disease Unit of the Osvaldo Cruz University Hospital (HUOC), at the University of Pernambuco (UPE). The selection of these individuals was based on the following criteria: 1) clinical tests for the characterization of the clinical forms established by the World Health Organization (WHO) (1); 2) positive serological tests for Chagas' disease; and 3) not having

been submitted to etiological treatment. The individual carriers of CF ($n = 19$, 33–82 years old; 8 females and 11 males), were selected by showing alterations in electrocardiogram and/or dilatation of the heart, absence of dilatation of the esophagus, absence of digestive complaints and positive serological results for *T. cruzi* infection. Individual carriers of IF ($n = 17$, 22–69 years old; 10 females and seven males), were selected by not having shown any type of cardiac and/or digestive alterations, but with positive serological results for *T. cruzi* infection (1). A group of non-infected Chagas' disease volunteers (NC) ($n = 19$, 21–50 years old; 14 females and seven males), was assembled for comparison with Chagas patients according to three criteria: 1) never having resided in an endemic area of Chagas' disease; 2) never having received a blood transfusion; and 3) having a negative serological result for Chagas' disease. The methods presented in this study were approved by the ethics committee of CPqAM/Fiocruz.

Obtaining the Mononuclear Cells of Peripheral Blood

A total of 40 mL of heparinized blood was mixed to phosphate buffered saline (PBS) pH 7.2 (1:2) and added to tubes containing Ficoll-Hipaque (Amersham Biosciences, Uppsala, Sweden) (1:3 of the blood-PBS mixture). After centrifuging (900 *g* for 40 min at 20°C), the PBMC was removed. The cells were washed two times by centrifuge (400 *g* for 10 min at 20°C) in medium RPMI 1640 (Sigma, St. Louis, MO) and counted in a Neubauer chamber. The cell concentrations were adjusted for the cell proliferation tests and to obtain culture supernatants.

Cell Proliferation Assay

Cellular suspensions (2×10^5 cells/well) were deposited in 96-well culture plates (Costar 3595; Corning Incorporated, Corning, NY) and stimulated with phytohemagglutinin (PHA) (Cultilab, São Paulo, Brazil) (2.5 µg/mL), Epi-Ag (25 µg/mL), and the Rec-Ags CRA or FRA (2 µg/mL) (after previous kinetic dose-responses), and without stimulation. The plates were incubated (37°C/5% CO₂) for 6 days (after previous kinetic time-response). At 16 hr before the end of incubation, 0.5 µCi of ³H-thymidine (Amersham Biosciences) was added. The material of the cell culture was collected through a semiautomatic cell harvester and deposited on glass-fiber paper (Skatron Instruments, Sterling, VA). The incorporation of ³H-thymidine was quantified following the emitted β radiation expressed in counts per minute (CPM). The stimulation index (SI) was calculated by dividing the mean CPM of the stimulated cultures by the mean CPM of the nonstimulated cultures.

Evaluation of the Cytokines of Culture Supernatants

Cell suspensions (5×10^5 cells/well) were deposited in culture plates of 48 wells (Costar 3548; Corning Incorporated) and stimulated with PHA (10 µg/mL), Epi-Ag (25 µg/mL), and Recs-Ags CRA or FRA (2 µg/mL) (after previous kinetic dose-response) and without any stimulation. The plates were incubated (37°C/5% CO₂) for 24 and 72 hr and 6 days (after previous kinetic time-response). Supernatants were collected after centrifugation and stored at –70°C. Then, 96-well microplates (Nalge Nunc International Corporation, Rochester, NY) were sensitized with the monoclonal anticytokine antibodies (TNF-α = 2 µg/mL, IL-4 = 2 µg/mL, IL-10 = µg/mL, and IFN-γ = 0.5 µg/mL) (R&D Systems, Minneapolis, MN) and incubated overnight at 4°C. The culture supernatants (50 µL/well), as well as standards (R&D Systems) were added in duplicate and incubated overnight at 4°C. The standards were added after serial dilution with factor 2 from the initial concentrations: TNF-α = 2,000 pg/mL; IL-4 = 8,000 pg/mL; IL-10 = 8,000 pg/mL; IFN-γ = 16,000 pg/mL in RPMI 1640 2% fetal bovine serum (FBS). The monoclonal anticytokine antibodies combined with biotin (TNF-α = 100 ng/mL; IL-4 = 12.5 ng/mL; IL-10 = 500 ng/mL; and IFN-γ = 125 ng/mL) (R&D Systems) were added for 2 hr at ambient temperature. Streptavidin-peroxidase (1:10,000) (Pharmingen, San Jose, CA) was then added at room temperature and the immunocomplexes were detected utilizing revealing solution (KPL, Gaithersburg, MD). The reaction was blocked with citric acid 0.2 M and the reading was carried out in a spectrophotometer (model 3550; Bio-Rad Laboratories, Inc., Vienna, VA) at 405 nm. The results were done as the mean of the duplicates ± standard deviation (SD).

Statistical Analysis

The Kolmogorov-Smirnov test evaluated the normality assumption of the data. To verify the existence of differences between stimulated and unstimulated cultures within each group studied, the Wilcoxon test was used for paired samples. To compare the difference between the groups, the Kruskal-Wallis test followed by the Mann-Whitney test were used, when a difference between existed. All conclusions were taken to a significance level of 5%. The software programs used were Excel 2000 and Statistical Package for Social Sciences (SPSS, Redmond, WA) version 8.0 (SPSS Incorporated, Chicago, IL).

RESULTS

The lymphoproliferation response expressed as an SI is shown in Table 1. Proliferative responses of the

TABLE 1. Lymphoproliferative response when faced with *T. cruzi* antigens and with mitogen*

	CF (n = 19)		IF (n = 17)		NC (n = 19)
	SI $\pm$ SD	P	SI $\pm$ SD	P	SI $\pm$ SD
CRA	2.96 $\pm$ 2.22	<0.01	2.99 $\pm$ 2.43	<0.01	0.91 $\pm$ 0.23
FRA	2.96 $\pm$ 2.66	<0.01	2.40 $\pm$ 0.93	<0.01	0.82 $\pm$ 0.25
EpiAg	6.15 $\pm$ 2.85	<0.01	7.59 $\pm$ 5.16	<0.01	0.77 $\pm$ 0.24
PHA	16.02 $\pm$ 9.99	>0.05	20.92 $\pm$ 16.68	>0.05	10.84 $\pm$ 8.75

*A total of 36 Chagas carriers of the clinical cardiac (CF) and indeterminate (IF) forms were evaluated. The means of the stimulation indices (SI) of the Chagas patients were compared to those of the non-Chagas individuals (NC).

SD, standard deviation; P, P value comparing CF and IF with NC; CRA, cytoplasmatic repetitive antigen; FRA, flagellar repetitive antigen; Epi-Ag, soluble epimastigote antigen; PHA, phytohemagglutinin.

Fig. 1. Detection of TNF- α in supernatant of PBMC cultures of Chagas patients and NC carriers after stimulation with *T. cruzi* antigens CRA, FRA, and soluble Epi-Ags of *T. cruzi*. Individual carriers of the CF (n = 19) and of the IF (n = 17) and NC individuals (n = 19). The points ($\circ$, $\square$, ∇) represent the mean of the duplication. The horizontal bars represent the arithmetic mean. The statistical differences are indicated in the figure with the P value.

T. cruzi antigens were verified after 6 days of cell cultivation. It was observed that both CF and IF individuals showed a greater antigen-specific response than the NC individuals after stimulation with CRA, FRA, and Epi-Ag. The mitogenic stimulation, used as a positive control, was significantly larger in Chagas' disease patients when compared to NC individuals.

High levels of TNF- α and IFN- γ cytokines were detected in the culture supernatants of PBMC of CF and IF individuals after cell stimulation with CRA or Epi-Ag. (Figs. 1 and 2). The production of TNF- α in PBMC cultures of CF (mean = 662.52 pg/mL $\pm$ SD = 294.01) and IF (mean = 652.16 pg/mL $\pm$ SD = 239.88) stimulated with CRA was higher when compared to the PBMC cultures of NC (mean = 386.60 pg/mL $\pm$ SD = 203.80). A similar result was observed in the stimulated cultures with Epi-Ag, that is, CF (mean = 478.97 pg/mL $\pm$ SD = 259.93) and IF (mean = 460.64 pg/mL $\pm$ SD = 270.72) (Fig. 1).

Significant values were found when comparing production of IFN- γ of CF (mean = 2,725.56 pg/mL $\pm$ SD = 2,786.60) and IF (mean = 1,821.22 pg/mL $\pm$ SD = 1,578.22) with NC (mean = 977.80 pg/mL $\pm$ SD = 2,087.44) individuals, when the cells were stimulated with CRA. In the cell cultures stimulated with Epi-Ag, the production of IFN- γ was also significant when compared to CF (mean = 5,749.14 pg/mL $\pm$ SD = 5,084.95) and IF (mean = 8,181.38 pg/mL $\pm$ SD = 4,683.94) with NC (mean = 600.67 pg/mL $\pm$ SD = 1,331.80) individuals (Fig. 2). However, no significant differences were found between CF and IF. Although increased production of IL-10 in supernatants of PMBC of Chagas patients (CF and IF) after stimulation with *T. cruzi* antigens has been manifested, above all with FRA (Fig. 3), there was no significant difference in this production with the group of NC individuals. The production of IL-4 after stimulation with *T. cruzi* antigens was not observed (data not shown).

Fig. 2. Detection of IFN- γ in supernatant of PBMC cultures of Chagas and NC carriers after stimulation with *T. cruzi* antigens CRA, FRA, and soluble Epi-Ags of *T. cruzi*. Individual carriers of the CF (n = 19) and of the IF (n = 17) and NC individuals (n = 19). The points ($\circ$, $\square$, ∇) represent the mean of the duplication. The horizontal bars represent the arithmetic mean. The statistical differences are indicated in the figure with the *P* value.

Fig. 3. Detection of IL-10 in supernatant of PBMC cultures of Chagas and NC patients after stimulation with *T. cruzi* antigens CRA, FRA, and soluble Epi-Ags of *T. cruzi*. Individual carriers of the CF (n = 19) and of the IF (n = 17) and NC individuals (n = 19). The points ($\circ$, $\square$, ∇) represent the mean of the duplication. The horizontal bars represent the arithmetic mean.

DISCUSSION

The immune system plays a crucial role in Chagas' disease pathology, possibly performing both in the control of the *T. cruzi* infection by limiting the severity of the disease, and promoting tissue damage in the recognized area of the parasite (37). This fact makes it difficult to understand the participation of the cell immune response, especially regarding the functions of cytokines in the development of Chagas cardiopathy. The relationship between the reactivity of T cells against the parasite and its antigens has thus been demonstrated, and the phenotypical and functional characterization of these cells has permitted the investigation of

their functions in the establishment of the pathological responses (individual carriers of serious forms) or protectors (individuals with indeterminate forms) (37). This study's purpose was to investigate the cellular immune response in this disease by evaluating the response of two recombinant antigens of the parasite and their correlation with the clinical forms of Chagas' disease. To this end, PBMCs of cardiac clinical form and indeterminate form carrier patients were stimulated in vitro with the Rec-Ags CRA and FRA of *T. cruzi* and total parasite extract.

Our results showed that both CF and IF individuals demonstrated specific and significant specific proliferative response against CRA and FRA, that is two to three

times less than the response observed for the total parasite extract (Table 1). These results were not surprising, first as it is known that there is a large variation in the cell response of these individuals, and second because it is related to the response to recombinant antigens. This variation in cell response against raw antigens has already been demonstrated by Morato et al. (16) where 30% of IF and 33.3% of CF Chagas patients showed low reactivity of PBMC to six antigens derived from different clones of *T. cruzi*. The decrease in lymphoproliferative response was also related in a longitudinal study of immune response carried out by Mosca et al. (14), where levels of parasitemia (evaluated after artificial xenodiagnosis) were correlated with the reduction in cell proliferation in individuals with IF, suggesting that, in patients with CF, a modulating mechanism could have been missing.

Barros-Mazon et al. (10) showed that cell proliferation resulting from the activation of soluble trypomastigote antigens was significantly greater in Chagas patients (both CF and IF) when the cultures were treated with indometacine, an inhibitor of prostaglandin synthesis, suggesting that cell responses in Chagas patients are negatively regulated by prostaglandins. It is known that macrophages and/or dendritic cells, among others, secrete cytokines, such as IL-10, or soluble mediators, such as the reactive intermediates of oxygen and nitrogen, that can increase or suppress proliferative cell response (38–40). In general, there is little information about the mechanisms that regulate the parasite-specific lymphocytic response in Chagas patients.

Little, however, is known about the specific response to isolated antigens of the parasite. In this study, high levels of TNF- α e IFN- γ were detected in Chagas patients after stimulation of PBMC with CRA or Epi-Ag. However, the cytokines levels were not different between the patients with CF and IF (Figs. 1 and 2).

Studies in patients with CF have revealed a Type 1 immune response, with elevated production of TNF- α and IFN- γ . Cunha-Neto et al. (41) studied the production of cytokines in supernatant cultures of T cells obtained in heart biopsies of eight CF patients. The results showed that the cells of seven of eight biopsies produced IFN- γ , six of eight produced TNF- α and three of eight produced IL-10. However, IL-4 and IL-12 were not detected. The presence of IFN- γ and TNF- α , in the absence of IL-4, indicates a pattern of Type 1 inflammatory response in individual carriers of Chagas cardiomyopathy. Despite the small number of individuals studied by Cunha-Neto et al. (41), this predominance of TNF- α and IFN- γ is in agreement with other previous studies (42,43).

High levels of IFN- γ are associated with the severity of cardiac disease (9) probably due to an exacerbation of the immune response, particularly in the cardiac fiber, promoting the destruction of these cells (9,44). TNF- α , however, has been shown to modulate the expression of adhesion molecules, thus participating in the inflammatory processes for impressing lymphocytes to the inflammation site and contributing to the progression of the local inflammatory reaction in Chagas cardiopathy (45).

In our study, although we found evidence of an increased production of IL-10 in PBMC supernatants of Chagas patients (CF and IF) after stimulation with *T. cruzi* antigens, especially with FRA, there was no significant difference in this production with the group of NC individuals (Fig. 3)

It is known that immunoregulating cytokines (IL-10, IL-4, and transforming growth factor [TGF]- β) are important for controlling infection by *T. cruzi* (46). The regulatory T cells have been described as a population of T cells that regulate both innate and adaptive responses, including the response to pathogens or to self antigens (47,48). However, they can contribute to the escape of *T. cruzi* from the immune system of the host and to the establishment of the chronic phase of the disease (46).

Monocytes/macrophages in PBMC, of individuals with the IF form of the disease, are the main cell producers of IL-10 after stimulation with trypomastigote and epimastigote lysate (9) and can be involved in the regulation of the immune response in Chagas' disease, modulating the expression and function of IL-12 and IFN- γ (3). The destructive process in the patients with CF could, for this reason, be due to a failure in the regulation of the T helper type 1 (Th1) response by IL-10. According to Gomes et al. (9), factors such as genetic characteristics of the host, changes in the immune system dependent on age, superposition of infections by other microorganisms and/or reinfection with *T. cruzi*, could be involved in this immunopathological process.

Furthermore, individuals with positive serological tests for the infection, and without electrocardiographic changes, residents of communities in Paraguay, showed elevated levels of IL-4, another cytokine secreted by T cells from type 2 (49), suggesting that repeated exposures to the parasite could have caused a different profile of cytokines. The results of this study showed that there was no production of IL-4 after stimulation with *T. cruzi* antigens, in agreement with recent studies (18,21,22,50), in which patient selection did not occur solely in an endemic area of the disease.

The differences between our results and those described by Pereira et al. (unpublished results), where a distinct profile of cytokines was shown between CF and IF individuals, could have been related, principally,

to the different methods used in the studies. The low levels of cytokines in the cultures and/or their rapid consumption by cell receptors could alter the level of detection of these factors through ELISA capture (51). The evaluation of the intracytoplasmic cytokines by flow cytometry is thus more representative, as the cytokines are imprisoned in the interior of the cell cytoplasm (52).

In resume, this study showed that CRA was capable of inducing a type Th1 immune response, with elevated production of TNF- α and IFN- γ in Chagas patients with CF and IF clinical forms. However, to fully understand the evolution of the clinical forms of the disease, cytokines secreted by PBMC of Chagas patients will be evaluated following flow cytometry involving individual residents of different endemic areas, where other strains of *T. cruzi* are found.

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