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Comparative analysis of cell phenotypes in different severe clinical forms of Chagas' disease

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1. ABSTRACT

The understanding of the role of the immune response in the development of gastrointestinal and cardio-digestive (CD) forms of Chagas disease has received little attention. In this paper, the commitment of each leukocyte population of peripheral blood to the production of IFN-gamma, TNF-alpha, IL-12, IL-4, IL-5 and IL-10 was studied in patients with the CD form of Chagas disease. The data show that cells from patients with the CD form of the disease have distinct cytokine profiles when compared with the other clinical forms of Chagas disease and suggest that eosinophils are the major source of cytokine production in this clinical entity. The data presented in this paper demonstrate that patients with CD form can be distinguished from patients with gastrointestinal or cardiac forms of the disease by the distinct cytokine profile of peripheral blood cells.

2. INTRODUCTION

Chagas' disease is caused by the protozoan *Trypanosoma cruzi* and is a major public health problem in Latin America. It is estimated that 18 million people are infected and that more than 100 million are at risk of infection (1). Chronic *T. cruzi* infection leads to different clinical forms of the disease, the most prevalent being the indeterminate clinical form, followed by the cardiac form or gastrointestinal disorders, which can occur simultaneously with cardiac pathology (2).

There are two hypotheses for the immunopathogenesis of Chagas' disease. The first proposes that the pathogenesis of the disease is due to autoimmune mechanisms and the second proposes that it is due to the immune response against the parasites in the tissues (3). Regardless of this mechanism, the immune response is a

major participant in the development of the severe forms of this disease. As a result, there has been intensive investigation of the role of the immune response in the development of Chagas' disease.

Among factors that might contribute to the disease development of different clinical forms of the disease are cytokines (4). Studies of the chronic phase of the disease revealed that TNF-alpha level is a predictor of heart failure (5) and that IL-2 level may be useful as an indicator for chemotherapeutic intervention (6). Levels of gene expression of IL-2, IFN-gamma, IL-4 and IL-10 detected by RT-PCR have been shown to be related to parasite exposure (7). IFN-gamma production is related to morbidity in the cardiac (CARD) clinical form, and IL-10 secretion is related to regulation of the immune response in indeterminate (IND) patients (8).

Previous data from our laboratory demonstrated that patients with the gastrointestinal (DIG) clinical form of the disease have a reduced percentage of circulating CD4+ cells (2). In both CARD and DIG clinical forms, the presence of cytotoxic cells expressing TIA-1+ lymphocytes has been described with CD8+ cells being present at high numbers in the heart tissue(9). However, significant differences between CARD and DIG patients were observed in the lymphocytes content of the tissues. In DIG patients a high percentage of CD4+ cells, eosinophils and mast cells were also observed (D'Avila Reis, personal communication).

The involvement of eosinophils in parasite cytotoxicity (10) and the development of myocardium lesions (11) has been documented in experimental models as well as suggested in human histological studies. In those studies the investigators focused mainly on the effect of eosinophil-derived factors, such as neurotoxin, peroxidase and major basic protein on development or establishment of lesions. However, analysis of the role of cytokines synthesized by these cells has not yet been done. Because some chagasic patients with the gastrointestinal form also develop cardiac disease, we asked the question of whether the immune response of these patients shows a mixed pattern of cellular and cytokine expression similar to those observed for the cardiac or digestive clinical forms of Chagas disease, respectively. To achieve this goal we determined the percentage of cells producing different cytokines in the peripheral blood leukocytes from patients with the Cardio-digestive (CD) as well as CARD and DIG clinical forms of Chagas disease.

3. MATERIAL AND METHODS

3.1. Study population

The study population consisted of 30 patients in the chronic phase of the infection all living in the endemic area of Posse, Goiás, Brazil or identified at the ambulatory of the Centro de Treinamento e Referência em Doenças Infecciosas e Parasitárias – CTR-DIP / Faculdade de Medicina - UFMG - Belo Horizonte/MG. Positive serology for Chagas' disease was determined by two or more tests (indirect immunofluorescence, enzyme-linked immunosorbent assay – ELISA, indirect hemagglutination, or complement fixation). Patients who agreed to participate in this study signed a written consent form and were subjected to a standard screening protocol that included medical history, physical examination, electrocardiogram, echodopplercardiography, laboratory and chest X-ray examinations, esophagogram, and barium enema, as required. None of the patients included in this study was undergoing chemotherapy for *T.cruzi* infection. The exclusion criteria included the presence of systemic arterial hypertension, diabetes mellitus, thyroid dysfunction, renal insufficiency, chronic obstructive pulmonary disease, hydroelectrolytic disorder, alcoholism, history suggesting coronary artery obstruction, rheumatic disease, or the inability for undergo the examinations. The groups of patients were classified according to the clinical form as cardio-digestive (CD, n=10), ages between 34 to 80 (5M-5F); digestive (DIG, n=16), ages between 22 to 68 (5M-11F), and cardiac (CARD, n=4) ages between 28 to 66 (1M-3F). The study protocol complied with the Helsinki Declaration and was approved by the Ethics Committee of the Centro de Pesquisas René Rachou from Fundação Oswaldo Cruz (FIOCRUZ-MG).

3.2. Culture of whole blood cells

Peripheral blood samples were collected into Vacutainers containing sodium heparin (Becton Dickinson, San Jose, CA), and 500µl aliquots were dispensed into individual 17 x 100mm polypropylene tubes (Falcon 2059 – Becton Dickinson). A control tube provided a baseline measurement of cytokines and received 500µl of RPMI 1640 (GIBCO – Grand Island, NY) plus Brefeldin A-BFA (Sigma Chemical Company – St Louis, MO), at a final concentration of 10µg/ml. Blood samples were incubated for 4 hours at 37^{o}C in a 5% CO_2 humidified incubator followed by treatment with a final concentration of 2mM EDTA (Sigma) and incubation at room temperature for 15 minutes.

3.3. Detection of cytokine positive cells

Cultured whole blood samples were washed with 6ml of FACS buffer containing 0.015M Phosphate Buffered Saline (PBS) 0.5% Bovine Serum Albumin (BSA), and 0.1% sodium azide, pH 7.2 (Sigma). All centrifugations were performed at 600-X g at room temperature for 7 minutes. After resuspending the cells in 1ml of FACS buffer, 200µl aliquots were dispensed into two 12 x 75 mm polystyrene tubes (Falcon 2052 – BD) and individually stained with the manufacturer recommended concentrations of monoclonal antibodies conjugated with flourescein isothiocyanate for the following cell surface markers (antibody clones are indicated in parentheses): CD4(RPA-T4), CD8(RPA-T8), CD14(M5E2), CD16(3G8) and CD19(HIB19). After incubation in the dark for 30 minutes at room temperature, the stained samples were treated by gently vortexing with 2ml of FACS Lysing Solution (BD) and re-incubated for an additional 10 minutes for erythrocyte lysis. The samples were centrifugated, the supernatant discarded and the cell pellet incubated with 2ml of FACS permeabilizing solution containing FACS buffer and 0.5% of saponin pH 7.2

Table 1. Percentage of cells/total leukocytes producing specific cytokines in CD, DIG and CARD groups

		EOS	LINF	LTCD4	LTCD8	NK Cells	LB	MO
CD	TNF-alpha	3.14 ± 2.65[1]	4.58 ± 6.62[1]	1.53 ± 2.44[1]	1.07 ± 2.46[1]	0.63 ± 0.67[1]	1.35 ± 2.20[1]	15.46 ± 20.82
DIG		4.85 ± 3.81	19.41 ± 10.85	7.08 ± 5.17	4.41 ± 4.49	3.87 ± 2.32	4.05 ± 3.92	53.72 ± 22.26
CARD		2.78 ± 3.29[1]	16.26 ± 5.08[1]	7.88 ± 2.12[1]	2.83 ± 1.39[1]	3.16 ± 1.11[1]	2.39 ± 1.40[1]	40.99 ± 7.61
CD	IL-12	3.10 ± 1.87[1]	0.76 ± 0.47[1]	0.47 ± 0.21[1]	0.29 ± 0.30[1]			3.22 ± 1.36
DIG		1.73 ± 1.36	1.52 ± 0.89	0.93 ± 0.64	0.59 ± 0.42			2.16 ± 1.88
CARD		0.43 ± 0.30[1]	2.78 ± 2.10[1]	1.52 ± 1.18[1]	1.26 ± 0.96[1]			1.75 ± 1.19
CD	IFN-gamma	2.46 ± 3.70[1]	1.07 ± 0.68[1]	0.48 ± 0.31[1]	0.24 ± 0.27[1]	0.35 ± 0.20		
DIG		2.66 ± 2.37	3.29 ± 1.42	1.33 ± 0.70	1.06 ± 0.68	0.90 ± 0.69		
CARD		0.59 ± 0.35[1]	4.56 ± 2.48[1]	2.13 ± 1.55[1]	1.66 ± 1.37[1]	0.77 ± 0.44		
CD	IL-4	4.09 ± 1.92[1]	2.55 ± 1.23[1]	0.97 ± 0.38[1]	0.53 ± 0.51	0.60 ± 0.43	0.45 ± 0.27	
DIG		5.28 ± 4.69	5.92 ± 2.24	1.77 ± 0.96	1.45 ± 0.76	1.22 ± 0.84	1.48 ± 1.07	
CARD		1.97 ± 0.92[1]	6.89 ± 2.39[1]	2.91 ± 0.84[1]	1.47 ± 0.83	1.12 ± 0.40	1.39 ± 0.62	
CD	IL-5	2.40 ± 1.65[1]	0.61 ± 0.33[1]	0.42 ± 0.22[1]	0.19 ± 0.20			
DIG		4.01 ± 2.76	1.69 ± 0.64	0.93 ± 0.56	0.76 ± 0.33			
CARD		1.12 ± 0.69[1]	3.01 ± 1.24[1]	2.04 ± 0.55[1]	0.97 ± 0.70			
CD	IL-10	2.03 ± 1.18[1]	1.08 ± 0.55[1]	0.45 ± 0.24[1]	0.23 ± 0.20[1]		0.40 ± 0.23[1]	1.84 ± 0.85
DIG		1.59 ± 1.16	3.52 ± 3.01	1.11 ± 0.77	0.68 ± 0.31		1.73 ± 2.95	1.21 ± 1.16
CARD		0.73 ± 0.52[1]	4.96 ± 4.48[1]	2.35 ± 2.24[1]	1.14 ± 0.93[1]		1.47 ± 1.34[1]	3.58 ± 2.91

Values of eosinophils (EOS), lymphocytes (LINF), CD4+ T lymphocytes (LTCD4), CD8+ T lymphocytes (LTCD8), NK cells, B lymphocytes (LB) and monocytes (MO) are expressed as % mean ± SD for each group. Numer 1 indicates higher values of each cytokine in LINF, LTCD4, LTCD8, NK Cells and LB of CARD patients, and the higher values of each cytokine in EOS of CD patients with a significant level of $p < 0.05$.

(Sigma) and kept in the dark for 10 minutes at room temperature. The samples were centrifuged, the supernatant was gently decanted, and 3ml of FACS buffer was added to each tube. Then the samples from each tube were incubated with 20µl of the monoclonal antibodies conjugated with phycoerytrin to TNF-alpha(HTNFR-M1), IL-12(C11.5), IFN-gamma(B27), IL-4(8D4), IL-5(JES3-39D10), or IL-10(JES3-19F1). The monoclonal antibodies had previously been diluted 1:50 in sterile FACS permeabilizing solution and distributed in a 96 well U-bottom microtiter plate (Thomas 9383-A90). The cell preparations were incubated in the dark for 30 minutes at room temperature and then washed with 150 µl of FACS permeabilizing solution followed by 200µl of FACS buffer. After been washed, the cell preparations were fixed in 200µl having FACS fix solution containing 10 g/l paraformaldehyde, 10.2 g/l sodium cacodylate and 6.63 g/l sodium chloride (Sigma), pH 7.2 and stored at 4°C in the dark and analyzed by flow cytometry within 24 hours.

A total of 30,000 events/tube were acquired using a FACScan® flow cytometer (B.D. Pharmingen, San Diego, CA). CELLQuest™ software provided by the manufacturer was used for data acquisition and analysis. Flow cytometric assessment of cytokine-positive eosinophils was carry out by single color immunophenotyping with FL-2/PE-labeled anti-cytokine mAbs. We used different gating strategies to analyze the cytokine profile of distinct leukocyte subpopulations. A specific gating procedure was based on autofluorescent cells using non-related FL-3 channel versus forward scatter (FSC) to identify eosinophils. Cytokine expressing eosinophils were identified using FSC versus FL2/anti-cytokine-PE dot plots. A confirmatory graph was also set up to identify the eosinophils on FSC versus side scatter (SSC) dot plot distributions confined to the region of cells with high granularity.

Identification of the other populations was performed by dual color immunophenotyping using surface marked anti-FITC and anti-cytokine-PE labeled mAbs. Initially, lymphocyte scatter gate was set up, using FSC versus SSC dot plots. Cytokine expressing cells were identified using FL1/anti-cell marker-FITC versus FL2/anti-cytokine-PE dot plots.

3.4. Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Witnney test provided by the software GraphPad Prism 3.0 (San Diego, CA). Differences were considered significant at a p value less than 0.05.

4. RESULTS AND DISCUSSION

In this study we performed a comparative analysis of cell and cytokine expression profiles in the peripheral blood of patients with various clinical forms of Chagas disease. Different cell populations were investigated and the commitment of these cells to the production of different cytokines was evaluated. The cytokines assessed included IFN-gamma, TNF-alpha, IL-12, IL-4, IL-5 and IL-10. Table 1 presents the data obtained when cytokine production by leukocytes was evaluated: eosinophils (EOS), lymphocytes (LINF), CD4+ T lymphocytes (LTCD4), CD8+ T lymphocytes (LTCD8), NK cells, B lymphocytes (LB) and monocytes (MO).

Table 2. Commitment percentage of EOS and LINF to producing cytokines in CD, DIG and CARD groups.

CELL TYPE	CYTOKINE	CD	DIG	CARD
LINF	TNF-alpha	17.29 ± 6.34	22.06 ± 10.23	26.57 ± 9.08[1]
EOS		19.99 ± 14.50[1]	5.35 ± 2.49	4.23 ± 4.91
LINF	IL-12	11.07 ± 8.37	27.28 ± 14.02	51.46 ± 25.07[1]
EOS		40.13 ± 17.11[1]	29.92 ± 18.65	8.77 ± 1.23
LINF	IFN-gamma	40.86 ± 19.92	50.84 ± 20.51	70.82 ± 23.16[1]
EOS		46.33 ± 27.97[1]	35.09 ± 17.38	23.84 ± 23.07
LINF	IL-4	29.72 ± 12.87	47.61 ± 15.63	68.56 ± 5.29[1]
EOS		45.31 ± 17.51[1]	36.45 ± 15.85	19.17 ± 4.32
LINF	IL-5	21.77 ± 7.83	35.00 ± 19.42	74.39 ± 8.63[1]
EOS		78.23 ± 7.83[1]	65.00 ± 19.42	25.61 ± 8.63
LIF		20.49 ± 5.56	50.64 ± 19.40	54.84 ± 23.60[1]
EOS	IL-10	38.96 ± 16.61[1]	27.52 ± 16.84	11.75 ± 3.82

Values of lymphocytes (LINF) and eosinophils (EOS) are expressed as % means ± SD for each group. Numer 1 indicates higher values of each cytokine in LINF of CARD patients, and the higher values of each cytokine in EOS of CD patients with a significant level of $p < 0.05$.

Analysis of TNF-alpha expression shows that CD patients have a low percentage of cells positive for this cytokine except for EOS where similar percentages of cells expressing this cytokine were observed in all groups of patients. No significant differences in TNF-alpha expression were observed between DIG and CARD patients. These results are interesting because they show that the expression of TNF-alpha in the CD form, where both pathologies occur, does not follow the same pattern of either DIG or CARD, suggesting that a distinct mechanism may be involved on the development of this clinical form of Chagas disease. We also observed that in CD patients, the levels of IL-12 expression by EOS are distinct from those in DIG and CARD patients. IL-12 has been previously suggested to be an important cytokine involved on the development of resistance to infection due to its stimulatory activity of IFN-gamma synthesis, an important activator of the parasiticidal effect of macrophages (12). In CD patients it seems to correlate with the induction of an inflammatory response by these cells.

One of the major cytokines that has been demonstrated to be important in the development of cardiac pathology is IFN-gamma (8). The data presented in table 1 show that T cells from CARD individuals have the highest percentage of cells expressing IFN-gamma followed by T cells from DIG and CD patients. Expression of IFN-gamma by EOS showed that CD and DIG patients have similar percentages of cells expressing this cytokine, where CARD patients have much lower percentage. It is also interesting to note that both IL-4 and IL-5 are significantly expressed by LINF in DIG and CARD patients, while EOS are the main sources of these cytokines in CD patients.

The last cytokine evaluated was IL-10. For this cytokine, we observed a pattern of expression similar to that of IFN-gamma in all three groups of individuals evaluated. These results suggest that there are distinct mechanisms of pathological development for the different clinical forms of Chagas' disease, supporting the previously suggested hypothesis that the immune response of individuals with the DIG clinical form of Chagas disease is distinct from that of individuals with the CARD form (11). In the CARD form, the roles of IFN-gamma in the induction of pathology and of IL-10 in the modulation of the immune response have been previously demonstrated (8). However, the fact that in the CD form the immune response seems to be distinct from that of both the CARD and DIG forms is surprising since these individuals have both pathologies.

Because it has been shown that EOS and LINF are the most frequent cell populations present in tissue lesions we further investigated the putative role of these cells in CD and CARD pathologies. Whereas table 1 shows the percentages of cells positive for the different cytokines, table 2 shows the commitment of these cell populations to the expression of the described cytokines. The percentage of commitment is crucial for understanding the putative role of each cell population in the development of pathology in Chagas disease since the percentage of commitment may correlate with cytokine levels. To determine the commitment of these cells to the synthesis of the different cytokines, we evaluated the percentage of LINF and EOS positive for the various cytokines in total leukocytes.

Table 1 shows that although MO are the main sources of TNF-alpha in all groups of chagasic patients (Table 1), in CD patients the percentage of EOS producing TNF-alpha is similar to the others groups. TNF-alpha is believed to be involved in amplifying inflammatory reactions with the risk of enhancing tissue damage (10). A substantial number of patients develop gastrointestinal disorders secondary to the lesions of the enteric nervous system; these lesions seem to be an essential element in the pathogenesis of the DIG clinical forms, which have both the parasite and the immune response as important components (11).

In our studies we observed that EOS of the CD group were more committed to cytokine secretion than the same cell population in other groups of chagasic patients (table 2). In fact, EOS are resident cells of the gastrointestinal tract and have been shown to be present in substantially higher numbers in other tissues (13). D'Avila Reis et al (personal communication) demonstrated by immunohistochemistry that the predominant cell type in the

gastrointestinal lesions is EOS. Similar results have also been obtained in studies related to Chron's disease where EOS have been shown to play significant role on the development of this pathology (14).

In contrast to the results from the CD patients, we observed that LINF from the CARD group were more committed to cytokine synthesis than LINF from the other groups of patients, including higher percentages of cells positive for IL-12 and IFN-gamma, cytokines that have been correlated with the development of severe heart disease (table 2). It is also important to note that a high percentage of LINF were also more committed to the synthesis of cytokines such as IL-4, IL-5 and IL-10. These data are in agreement with those previously published by our group, suggesting the importance of LINF in inducing a severe inflammatory response in CARD patients, attributable to a favorable balance towards an inflammatory response (8). While in CARD patients the major commitment to synthesis of IFN-gamma and IL-12 was of LINF, in CD patients EOS were the cells most committed to the synthesis of these cytokines, suggesting that the mechanism involved in these two clinical forms of the disease may be related to the source of the cytokines.

Our results indicate that depending on tissue location of the parasite *T. cruzi*, a significantly different immunopathological mechanism may be induced. It is important to point out that IFN-gamma and IL-5 also seem to be the major cytokines involved in CARD and DIG patients, promoting tissue damage and traffic of EOS (8, 10); even though, the sources of these cytokines are distinct. In summary, these results show that in the development of the CD clinical form of Chagas' disease EOS are highly committed to the secretion of pro-inflammatory cytokines that may be driving the pathology in these individuals.

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Footnote: Written informed consent was obtained from patients according to the guidelines of the Ethics Committee of Centro de Pesquisas René Rachou – Fundação Oswaldo Cruz (FIOCRUZ-MG).

Key Words: Chagas' disease, Cytokine, Clinical forms, Parasite, Disease, Blood, Cell

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