LYMPHOCYTE SUBPOPULATIONS AND NEUTROPHIL FUNCTION IN CHRONIC HUMAN CHAGAS' DISEASE

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SUMMARY

The absolute numbers of total leukocytes, lymphocytes, T cells, helper/inducer, suppressor/cytotoxic and B cells were decreased in the peripheral blood of patients with chronic Chagas' disease. Since antilymphocyte antibodies were present only in a minority of patients they probably cannot account for the abnormalities in lymphocyte subsets. Patient neutrophils stimulated with endotoxin-treated autologous plasma showed depressed chemotactic activity and this seems to be an intrinsic cellular defect rather than plasma inhibition. Random migration of neutrophils was normal. Reduction of nitroblue tetrazolium by endotoxin-stimulated neutrophils was also decreased. These findings further document the presence of immunosuppression in human Chagas' disease. They may be relevant to autoimmunity, defense against microorganisms and against tumor cells at least in a subset of patients with more severe abnormalities.

KEY WORDS: Chagas' disease; Leukopenia; Lymphopenia; lymphocyte subpopulations; Chemotaxis; NBT test; Antilymphocyte antibodies.

INTRODUCTION

Infection by **Trypanosoma cruzi** has been associated with a non-specific suppression of the immune response in animals[7, 26] and in humans[4, 8, 10, 33, 35, 36]. Depression of cell mediated skin reactivity to dinitrochlorobenzene[33] and of CD4/CD8 cell ratio and neutrophil function[35] was observed in patients with acute Chagas' disease. In addition, in the chronic forms of the disease there is an impairment of the antibody response to sheep erythrocytes[9] and to typhoid vaccine[8], and a decrease of the antibody dependent cellular cytotoxicity (ADCC) to chicken erythrocytes[36] and of the skin reactivity to tuberculin[4]. The mechanism of immunosuppression in trypanoso-miasis is poorly understood[7] and an inbalance between helper and supressor T cells was suggested[9, 16] from animal studies. However, no direct evidence has been presented to support this hypothesis in human Chagas' disease. Indeed, in the only study in which T cell subsets in chronic Chagas' disease patients were measured, inconclusive results were obtained with remarkable sex differences[15].

Monocyte, neutrophils and eosinophils from normal individuals[17, 18, 23, 27] or from chagasic patients[27, 36] are able to lyse antibody-coated **T. cruzi** probably following ingestion of the para-

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site[30, 31]. In contrast, monocytes from chronic chagasic patients were not able to lyse **Candida pseudotropicalis** following phagocytosis[11]. However, there are few studies of other functions of phagocytic cells from chagasic patients[13].

The purpose of the present investigation was to evaluate lymphocyte subpopulations, including T helper and T supressor/cytotoxic cells, neutrophil chemotaxis and nitroblue tetrazolium (NBT) reduction in chronic chagasic patients.

MATERIAL AND METHODS

Subjects: Lymphocyte subpopulations and neutrophil function were evaluated in the peripheral blood of 34 patients with chronic forms of Chagas' disease and 34 male control blood donors. The diagnosis was established by clinical (cardiomyopathy or mega syndromes) and serological (complement fixation or imunofluorescence reaction to **T. cruzi**) findings. All patients included in this clinical investigation had been admitted to the University Hospital of the School of Medicine of Ribeirão Preto and were in good nutritional status and did not display any severe impairment of physiological functions due to Chagas' disease. They did not report the use of drugs known to interfere with the immune system.

Total leukocyte and lymphocyte counts: The absolute number of total lymphocytes was estimated in whole blood from total leukocyte counts performed in a Coulter counter and differential lymphocyte counts performed in Leishman stained smears.

Preparation of lymphocytes for quantitation of subpopulations: Lymphocyte suspensions were obtained by diluting (1:3) heparinized blood samples in phosphate buffered saline (PBS) followed by centrifugation at 400 g for 30 min at 25°C on a Ficoll-Hypaque gradient (specific gravity 1.076)[5]. Cells remaining in the interface were washed three times and resuspended in TC 199 (Flow Laboratories) containing 20% fetal calf serum (FCS) (Difco) at 8-16 x 10^{6}/ml. The monocytes were excluded by aderence to plastic Petri dishes for 1 h at 37°C. Non-adherent cells were centrifuged and resuspended in RPMI 1640 with 5% of FCS at 5 x 10^{6}/ml.

B cell quantitation: Lymphocytes were washed three times with PBS at 37°C to remove passively absorbed immunoglobulins. Aproximately 2 x 10^{6} cells were mixed with 0.1 ml of fluorescein-conjugated goat serum anti-human immunoglobulins (Behringwerk, Marburg, West Germany) previously diluted to 1:100 in PBS containing 0.2% sodium azide. The resulting suspension was then kept in an ice bath for 30 min. After three further washes with cold PBS-azide solution, the cells were resuspended in a small volume of FCS. Smears were prepared, fixed in methanol for 10 sec, washed in PBS and mounted with buffered glycerol. The total number of cells present in each microscope field was initially determined by phase contrast under visible light. The number of fluorescent cells in the same field was then estimated under UV ilumination. The percentage of B cells was determined by counting 100 lymphocytes in each sample.

T cell subset assay: Two hundred μl of the lymphocyte suspension were incubated with 5 μl of appropriatedly diluted monoclonal antibody (OKT3 or anti-CD3, OKT4 or anti-CD4, OKT8 or anti-CD8, Ortho Pharmaceutical Company, Raritan, New Jersey) at 4°C for 30 min, with shaking every 10 min. Cells were washed twice in RPMI 1640 containing 5% FCS and resuspended in 100 μl of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Nordic Imunology, Tillburg, The Netherlands) diluted to 1:20 in PBS. Following 30 min incubation at 4°C, the cells were washed twice with RPMI 1640 and the sediment was resuspended in a drop of FCS. Smears were prepared, fixed in methanol, washed for 30 sec in PBS and mounted in one drop of buffered glycerol. Analysis was performed immediately as described for B cells.

Antilymphocyte antibodies: Antilymphocyte antibodies were evaluated in chagasic sera in 3 different ways: by microlymphocytotoxicity, by immunoflourescence in a flow cytometer (FACS) and by antibody-dependent cell cytotoxicity (ADCC). The microlymphocytotoxicity test was modified from AMOS et al.[3]. Briefly, one microliter of a suspension containing 5,000 lymphocytes was incubated for 30 minutes with 1 μl of patients serum in a Terasaki microplate, washed twice and then incubated with 4 μl of rabbit complement for 2 h at room temperature.

Positive reactions were revealed by cell death after staining with eosin Y or trypan blue. Sera from chagasic patients were tested against their own cells (auto crossmatch) and against a panel of cells from 40 to 100 individuals. The immunofluorescent test was based in the FACS crossmatch test[14]. Briefly, undiluted and 1:10 chagasic sera was incubated with a pool of lymphocytes from 4 normal donors for 20 min at room temperature, washed 3 times and incubated with fluoresceinated goat F (ab')$_2$ anti-human immunoglobulin (Tago, Burlingame, CA) for 30 min at 4°C. After washing twice, cells were ressuspended in 0.25 ml of PBS containing 0.1% sodium azide and analyzed in a Facsan flow cytometer (Becton Dickinson, Montain View, CA). Histograms displaying cell number on the vertical axis and fluorescent intensity on the horizontal axis were generated after gating on the lymphocyte population and counting 10,000 cells. The histograms show two peaks: a large low intensity peak comprising non-B (mostly T) cells and a small high intensity peak comprising B cells. A shift to the right of at least 10 channels of either T or B peak was considered a positive reaction. ADCC reaction was measured by a ^{51}Cr release assay, using two human B cell lines as targets, normal PBL as effector cells and sera from chagasic patients diluted at 1:50 as sensitizing agent.

NBT test: The modified stimulated NBT test was carried out as described previously[12]. Briefly, 0.1 ml of peripheral blood collected in EDTA was mixed with equal volume of NBT solution (0.1% NBT Sigma in PBS) plus 0.05 ml of endotoxin solution (1 mg/ml of lipopolysacharide B from **E. coli** 0127:88 Difco in PBS) in a glass tube (12 x 75 mm). The mixture was incubated at 37°C for 15 min, the tube was centrifuged and smears were made of the cell pellet. The smears were stained with Leishman, 100 neutrophils were counted and only those with large black formazan deposits in the cytoplasm were scored as positive.

Neutrophil chemotaxis: Neutrophil chemotaxis was evaluated by a modification of the leading front method of ZIGMOND & HIRSH[37] as described by AKENZUA & AMIENGHEME[2]. Briefly, 200 μl of leukocyte suspension (2 x 10^6/ml in TC 199 Flow) isolated by Dextran sedimentation from peripheral blood were placed inside

plastic test tube stoppers and gently inverted on to 13 mm diameter Millipore membrane (Bedford, MA). with pore diameter of 3 μm. The membranes were placed on 13 mm diameter AA discs (Millipore), soaked with chemotactic factor (12.5 μg of endotoxin in 25 μl of autologous plasma plus 225 μl of medium). After 30 min incubation at 37°C in a moist box, the membranes were removed, washed with medium, fixed with isopropyl alcohol, stained in hematoxylin, cleared in xylene and mounted on slide with Canada balsam. To measure the distances migrated by neutrophils, the membranes were examined under a x100 objective. The distance migrated by neutrophils was the average of differences, measured on five different fields, between the micrometer reading on the top of the membrane and on a plane where only five cells were left in view. Random migration was the distance migrate by neutrophils in the presence of medium only. Chemotactic index was the difference between the distance migrated by neutrophils stimulated by endotoxin-treated plasma and by medium. Crossed incubations between plasma and cells from controls and patients were performed to investigate the cause of low chemotactic index in some patients.

Quantitation of serum immune complexes: An enriched immune complex serum fraction was obtained by a polyethylene glycol (PEG) precipitation method, according to ZUBLER et al.[38] with minor modifications. Briefly, one volume of serum was mixed with three volumes of isotonic borate-EDTA buffer pH 8.4 containing 4% PEG (MW 6,000). The determination of C1q-precipitins was carried out as described by LEVINSON & GOLDMAN[21]. Human gamma globulin purified by passage through DEAE-cellulose chromatography and heat-aggregated by incubation at 63°C for 20 minutes (HAGG) was utilized for calibration of reference curves of absorption nephelometry in a double beam nephelometer (Beckman model 35, USA). The results are reported as μg/ml equivalents HAGG.

Statistical analysis: Statistical analysis of lymphocyte subpopulations was performed by Student's t test. The distribution of B lymphocyte number and of CD4/CD8 cell ratio were submitted to logarithmic transformation in order to approximate a normal distribution. Statistical analysis of NBT test and chemotaxis was performed simultaneously by the Hotelling's T^2

test. A value of p < 0.05 was taken as statistically significant.

RESULTS

Leukocyte counts: Patients with chronic Chagas' disease had significantly lower leukocyte counts (6.45 ± 1.77 x 10^9/L, mean ± SD) than normal controls (7.44 ± 1.97 x 10^9/L).

Lymphocyte subpopulations:

The absolute number of total lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$, and B lymphocytes and the percentage of B lymphocytes were significantly reduced in patients with chronic Chagas' disease as compared with controls (Table 1). The CD4/CD8 cell ratio and the percentages of total lymphocytes and T cell subpopulations were similar in patients and controls. The distribution of CD4/CD8 ratio showed a wider range of variation on chagasic patients (0.66 to 7.90) than in normal controls (1.32 to 4.43) (Fig. 1). No significant difference was shown for the number or percentage of total lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$ or B cells or in the CD4/CD8 ratio between male and female chagasic patients (Table 2).

Antilymphocyte antibodies:

When tested against their own cells by mycrolymphocytotoxicity, the presence of antilymphocyte antibodies was not detected in any of 15 patients studied. When tested against a panel of lymphocytes of different individuals, an abnormal reactivity (positivity against > 10% of individuals) was detected in 6 out of 31 patients. Three of these 6 patients could have been allosensitized by multiple pregnancies or transfusions. Four of these patients showed specific sensitization against the antigen HLA B14. The FACS crossmatch against a pool of lymphocytes was positive in 3 out of 15 patients tested. Two of these 3 patients could have been sensitized through multiple pregnancies. The ADCC test revealed the presence of antilymphocyte antibodies in the serum of 2 chagasic patients, one of them being a multiparous.

Stimulated NBT test and neutrophil chemotaxis:

The percentage of neutrophils with formazan (reduced NBT) granules in the cytoplasm was 78.5 ± 14.4% (mean ± 1 SD) in controls and 70.8 ± 19.7% in patients (p = 0.069). The chemotatic index was 43.7 ± 13.3 μm in controls and 34.9 ± 18.3 μm in patients (p = 0.027). The combined statistical analysis of NBT and chemotaxis showed that patients with Chagas' disease had decreased values compared to normal controls (p = 0.009). Six patients showed low chemotactic index and five, low NBT reduction; however, no-

TABLE 1

Lymphocyte subpopulations in chronic human Chagas' disease

Lymphocytes	Number (x 10^9/L)		Percent	
	Controls n = 34	Patients n = 34	Controls	Patients
Total	2.22 ± 0.58	1.68 ± 0.67*	30.9 ± 7.4	27.1 ± 11.0
$CD3^+$	1.66 ± 0.43	1.27 ± 0.54*	75.1 ± 6.1	75.1 ± 6.2
$CD4^+$	1.18 ± 0.32	0.93 ± 0.41*	53.3 ± 8.8	55.2 ± 9.1
$CD8^+$	0.60 ± 0.25	0.47 ± 0.27*	27.6 ± 6.8	27.3 ± 9.5
$CD4^+/CD8^+$	0.73 ± 0.33[a] (2.18)[b]	0.75 ± 0.48 (2.38)		
B	-1.4 ± 0.49[a] (0.28)	-1.9 ± 0.06* (0.17)	12.7 ± 4.3	10.4 ± 3.8*

[a] Data are mean and SD after logarithmic transformation ($\log_e$) to correct for skewness.
[b] Means reexpressed in original units.
* Significantly different from controls, p < 0.05.
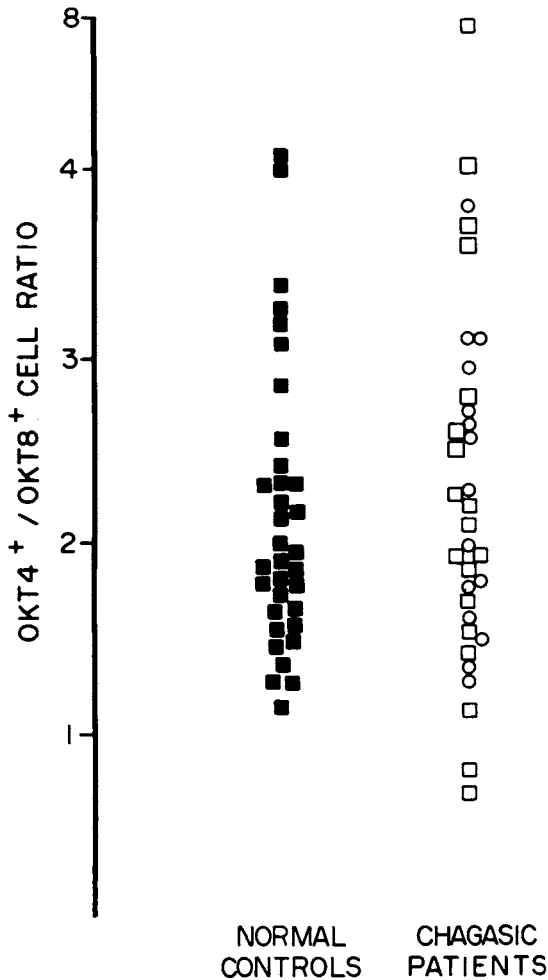n = number of cases.

Fig. 1 — Distribution of CD4/CD8 cell. ratio in male (square) and female (circles) normal controls (black) and chagasic patients (white). The monoclonal antibodies OKT4 and OKT8 stain respectively CD4 and CD8 positive lymphocytes.

TABLE 2

Lymphocyte subpopulations in male and female chagasic patients

Lymphocytes		Male n = 19	Female n = 15
Total	x 10⁹/1	1.62 ± 0.61*	1.77 ± 0.76
	%	28.3 ± 9.2	26.5 ± 12.5
CD3⁺	x 10⁹/1	1.20 ± 0.46	1.37 ± 0.62
	%	74.3 ± 6.7	76.3 ± 5.5
CD4⁺	x 10⁹/1	0.87 ± 0.38	1.01 ± 0.45
	%	53.5 ± 10.4	57.4 ± 6.8
CD8⁺	x 10⁹/1	0.44 ± 0.23	0.50 ± 0.32
	%	28.3 ± 10.8	25.9 ± 7.6
CD4⁺/CD8⁺		2.46 ± 1.62	2.28 ± 0.78
B	x 10⁹/1	0.14 ± 0.07	0.20 ± 0.10
	%	9.4 ± 4.2	11.6 ± 2.8

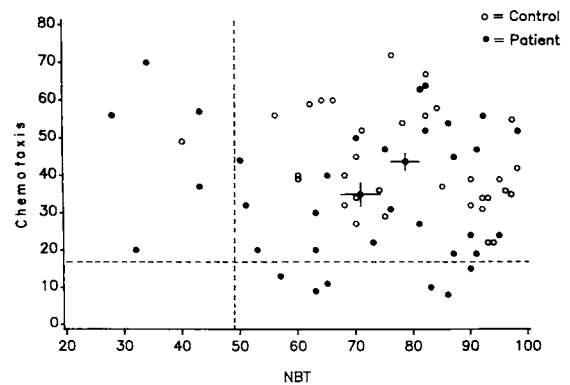* mean ± standard deviation

n = number of cases

Fig. 2 — Combined plot of endotoxin-stimulated chemotaxis and NBT reduction by neutrophils of normal controls and chronic chagasic patients. Chemotaxis is expressed in μm of migration and NBT index is the percentage of neutrophils with formazan granules in the cytoplasm. The means for each variable are indicated by the targeted lines. Dashed lines indicated lower limits of normal for each test. Combined statistical analysis by the Hotelling T^2 test showed that the group of chagasic patients had lower chemotaxis and NBT reduction than controls (p = 0.009).

TABLE 3

Neutrophil chemotaxis in some patients with chronic Chagas' disease

Patient	CN + CP	PN + PP	CN + PP	PN + CP
1	39*	22	46	36
2	36	17	35	18
3	72	20	43	8
4	45	26	31	28
5	24	8	32	16

CN — control neutrophils   * migration (micra)

CP — control plasma

PN — patient neutrophils

PP — patient plasma

ne showed abnormal results in both tests (Fig. 2). In crossed experiments, plasma from patients with low chemotaxis were able to generate normal chemotactic stimulus for control neutrophils. However, the incubation of neutrophis from 3 out 5 patients with control plasma did not revert the low chemotactic index (Table 3). Neutrophil random migration was similar in controls (33.6 ± 8.9 μm) and patients (32.4 ± 6.6 μm).

Serum immune complexes:

Quantitation of serum immune complexes by C1q precipitins showed similar results in con-

trols (179.8 ± 23.1 μg/ml) and patients (188.6 ± 51.1 μg/ml).

DISCUSSION

In this study we observed that patients with chronic Chagas' disease had a decrease in the absolute number of total leukocytes, total lymphocytes, T3[+], T4[+], T8[+] and B lymphocytes in the peripheral blood and a normal ratio of helper to suppressor cells. In a previous report, we described a decrease in the total number of lymphocytes which was associated with the presence of antibodies directed to mouse lymphocytes in all patients[36]. However, in the present study we could not detect autoantilymphocyte antibodies in any patient tested and we found alloantibodies in only a minority. Interestingly, some patients demonstrated alloantibodies against the antigen HLA-B14. These antibodies could have been generated by cross-reaction existing between the HLA-B14 antigen and the **T. cruzi.**[24]. Thus, sera from chagasic patients seem to contain antibodies that react to mouse lymphocytes but not to human lymphocytes. In this study, we used the most sensitive methodology available to detect human antilymphocyte antibodies, such as the FACS crossmatch technique and ADCC, besides the complement-dependent microcytotoxicity, but we cannot exclude the possibility that techniques even more sensitive may detect the antibodies. In fact, immunoblotting analysis appear to be demonstrating antilymphocyte antibodies in the sera of most chagasic patients (W. Savino, personal communication). Combination of antibody-mediated neutropenia and lymphopenia is observed in other diseases with autoimmune features such as systemic lupus erythematosus and could be occuring in chagasic patients as well. Alternatively, cellular autoimmune mechanisms known to be operating in **T. cruzi** infection[32] might be responsible for leukocyte destruction in Chagas' disease. Furthermore, basal plasma cortisol levels were normal in chagasic patients[19] and therefore cannot account for the abnormalities in lymphocyte subpopulations. Thus, the leukopenia of chronic Chagas' disease is caused by an yet unknown metabolic or immunological mechanism.

Normal values for the percentage and absolute number of T and B lymphocytes[20, 25], for the percentages of total T, CD3^{+}, CD4^{+}, CD8^{+} cells and of CD4/CD8 ratio[15], decreased percentages of T and B lymphocytes associated with normal number of total lymphocytes[8] and reduced number of total T and B lymphocytes[4] were previously described in patients with Chagas' disease. Recently, GATASS et al.[15] described several abnormalities of T cell subpopulations in chronic patients which included a wider range of CD4/CD8 ratio, with males exhibiting lower values of the ratio than females, and increased percentage of CD4^{+} cells and decrease of CD8^{+} cells in females compared with male patients. In a previous report, we described a reduction of the CD4/CD8 ratio in a female patient with acute Chagas' disease[35]. Although we confirmed the wider range of variation of CD4/CD8 ratios in chronic chagasic patients in the present study, we did not observe the sex differences in lymphocyte subpopulations as reported by GATASS et al.[15]. The origin of these different results could not be determined precisely, but it may be due to the selection of patients. Although we have excluded from our group every patient with compromised general and nutritional status or severe cardiac, oesophageal or intestinal abnormalities or using any drug potentially affecting the immunologic system, it is possible that our hospital population reflects a selection of a distinct subset of patients from that of previous studies. Regional differences among groups of patients should also be considered. The relevance of the lymphocyte subpopulations abnormalities which we described here to the immunosuppression previously described in Chagas' disease[8, 10, 33] is unclear since we did not detect an immunoregulatory inbalance in the helper/suppressor cell ratio. These abnormalities may be relevant to the increased incidence of malignant neoplasms observed in patients infected with **T. cruzi**[22, 29]. In fact, mice with chronic chagasic infection showed suppression of tumor specific T lymphocyte response[28].

Regarding neutrophil function, patients with chronic Chagas' disease exhibited a significant decrease in the chemotactic activity and in the reduction of NBT, both functions stimulated **in vitro** by bacterial endotoxin. We were not able to demonstrate in crossed experiments with control plasma and cells any inhibitory activity of patients' plasma on chemotaxis. Furthermore, we could not detect in the serum of chagasic patients increased levels of immune

complexes, a factor known to depress leukocyte chemotaxis. In addition, neutrophils from some patients did not revert the low chemotactic index when incubated with normal plasma. These findings point out to an intrinsic chemotactic defect in patients neutrophils, of unknown origin. Likewise, FERREIRA et al.[13] showed depressed endotoxin stimulated chemotactic activity of blood monocytes from patients with chronic Chagas' disease and did not find inhibitory influence of patient sera on chemotaxis. In our study, random migration of patient neutrophils was intact, suggesting that the chemotactic defect is at the level of interaction between the cell and the chemotactic stimulus rather than in the cellular movement apparatus. In a previous report, we found severe deficiency of neutrophil chemotaxis and NBT reduction in one patient with acute Chagas' disease[35]. Moreover, **in vivo** leukocyte chemotaxis was progressively decreased in mice with acute **T. cruzi** infection[1]. The reduction of NBT stimulated by endotoxin was also depressed in chagasic patients and this metabolic defect may compromise their ability to fight several types of infection including the trypanosomiasis. In fact, the NADPH/oxidase system, probed by the NBT test, may be involved in the destruction of epimastigotes[6] and amastigote[34] forms of **T. cruzi**. In addition, killing of **Candida pseudotropicalis** by monocytes was decresed in chagasic patients[11]. On the other hand, neutrophils from patients with chronic Chagas' disease can kill antibody-coated **T. cruzi** as efficiently as normal controls in an **in vitro** ADCC reaction[27, 36].

The abnormalities in neutrophil function and leukocyte numbers in the group of chagasic patients were moderate, but it is apparent from Fig. 2 for example that a subset of patients had a more severe dysfunction. In fact, values below the normal range of chemotaxis were observed in 6 patients and of NBT test in 5 patients. These patients may have clinical problems in handling infectious agents, including the **T. cruzi**, and tumor cells. Uncontrolled clinical observation points out that chagasic patients are not susceptible to frequent or severe infections, but this subject has not been carefully investigated in humans. On the other hand, there are suggestions of increased incidence of cancer in human Chagas' disease[22, 29]. Increased susceptibility to infections may be a problem for a subset of patients

with more defective neutrophil function and decreased leukocyte numbers. Likewise, the quantitative abnormalities in lymphocyte subpopulations may play a role in the asymptomatic immunosuppression of chronic chagasic patients and may be related to the increased susceptibility to neoplasms in a subset of patients exhibiting more severe lymphopenia.

RESUMO

Subpopulações linfocitárias e função neutrofílica na doença de Chagas humana.

Números absolutos de leucócitos e linfócitos, de células T totais, indutoras/auxiliares, supressoras/citotóxicas e de células B estavam diminuídos no sangue periférico de pacientes com doença de Chagas crônica. Como anticorpos antilinfocitários estavam presentes em apenas uma minoria de pacientes, eles provavelmente não são responsáveis pelas anormalidades das subpopulações de linfócitos. Neutrófilos de pacientes estimulados por plasma autólogo tratado por endotoxina mostravam atividade quimiotática diminuída que deve ser devida a um defeito celular intrínseco e não a inibição plasmática. A migração aleatória dos neutrófilos estava normal. A redução do corante "nitroblue tetrazolium" (NBT) por neutrófilos estimulados por endotoxina também estava diminuída nos pacientes. Estes achados estendem a documentação da imunossupressão na doença de Chagas humana. Eles podem ser relevantes para autoimunidade e para defesa contra microorganismos e células tumoráis, pelo menos em um subgrupo de pacientes com anormalidades mais pronunciadas.

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