

## PLASMA LEVELS OF TUMOR NECROSIS FACTOR- $\alpha$ IN PATIENTS WITH VISCERAL LEISHMANIASIS (KALA-AZAR). ASSOCIATION WITH ACTIVITY OF THE DISEASE AND CLINICAL REMISSION FOLLOWING ANTIMONIAL THERAPY

Reinaldo SALOMÃO (1), Adauto CASTELO FILHO(1), Iara Marques de MEDEIROS(2) & Miguel Angel SICOLO(2)

### SUMMARY

Evaluation of TNF-alpha in patients with Kala-azar has drawn increasing interest due to its regulatory role on the immune system, in addition to its cachetizing activity. The objective of this study was to examine the association between plasma levels of TNF-alpha, measured by immunoreactivity (ELISA) and bioactivity (cytotoxicity assay with L-929 cells), and clinical manifestations of visceral leishmaniasis. Plasma samples from 19 patients with Kala-azar were obtained before, during and at the end of antimonial therapy. TNF-alpha determinations was done by using the cytotoxicity assay (all patients) and the enzyme-linked immunoassay (ELISA - 14 patients). A discrepancy between results obtained by ELISA and cytotoxicity assay was observed. Levels of circulating TNF-alpha, assessed by ELISA, were higher in patients than in healthy controls, and declined significantly with improvement in clinical and laboratory parameters. Plasma levels before treatment were  $124.7 \pm 93.3$  pg/ml (mean  $\pm$  SD) and were higher than at the end of therapy  $13.9 \pm 25.1$  pg/ml (mean  $\pm$  SD) ( $p = 0.001$ ). In contrast, plasma levels of TNF-alpha evaluated by cytotoxicity assay did not follow a predicted course during follow-up. Lysis, in this case, might be not totally attributed to TNF-alpha. The discrepancy might be attributed to the presence of factor(s) known to influence the release and activity of TNF-alpha.

**KEYWORDS:** Cytokines; TNF- $\alpha$ ; Visceral Leishmaniasis (Kala-Azar)

### INTRODUCTION

Leishmaniasis is a prevailing disease in tropical and subtropical areas. In 1990, the World Health Organization estimated that 350 million people were infected, and 12 million developed the disease<sup>17</sup>.

The wide spectrum of clinical manifestations is related to the *Leishmania* species and the immune status

of the infected host, and includes cutaneous, mucocutaneous and visceral diseases<sup>18</sup>.

Seropidemiological surveys have shown that one out of six individuals exposed to *Leishmania (Leishmania) donovani* will eventually develop disease<sup>3</sup>. Of interest, some of the exposed people will only have self-

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Informed Consent was obtained from patients or their parents or guardians before enrollment in this investigation.

(1) Division of Infectious Diseases. Escola Paulista de Medicina, Rua Botucatu 740, 04023-062 São Paulo, SP, Brasil.

(2) Division of Infectious Diseases. Universidade Federal do Rio Grande do Norte, Rua Cônego Monte s/n, 59000 Natal, RN, Brasil.

**Correspondence to:** Reinaldo Salomão, Division of Infectious Diseases, Escola Paulista de Medicina, Rua Botucatu 740, 04023-062 São Paulo, SP, Brasil.

limited subclinical disease, whereas others will progress towards a full-blown Kala-azar<sup>3</sup>, probably depending on the pattern of immune response at the time of infection<sup>7</sup>.

TNF-alpha is a cytokine produced mainly by macrophages in response to a wide variety of infectious agents<sup>5,26</sup>. TNF-alpha plays an important role in host defense against infections caused by intracellular microorganisms such as *Bacillus Calmette-Guérin*<sup>14</sup>, *Chlamydia trachomatis*<sup>28</sup> and *Listeria monocytogenes*<sup>16</sup>, and it is considered to have a pivotal role in the pathophysiology of endotoxic shock<sup>6,11,23</sup> and cerebral malaria<sup>12</sup>.

Evidences based on experimental cutaneous leishmaniasis suggest a protective role for TNF-alpha. This cytokine has been shown to be produced in lymph nodes of a resistant strain (C3H) of mice infected with *L. (L.) major*, whereas no such production could be observed in susceptible mice (Balb/c)<sup>22</sup>. Administration of recombinant TNF-alpha to susceptible mice caused reduction of cutaneous lesions, while administration of antibodies anti-TNF-alpha led to progression of lesions<sup>15,22</sup>. In experimental visceral leishmaniasis TNF-alpha seems to be critical both for resistance to *L. (L.) donovani* and resolution of infection<sup>25</sup>.

Evaluation of TNF-alpha in patients with Kala-azar has drawn increasing interest due to its regulatory role on the immune system, in addition to its cachetizing activity. Patients with visceral leishmaniasis often present weakness, weight loss, and anemia, symptoms that could be mediated by TNF-alpha<sup>24</sup>. Circulating TNF-alpha has been detected in patients with visceral leishmaniasis<sup>20,21</sup> and its presence has been related to disease activity<sup>4</sup>.

The main objective of the present work was to examine the association between plasma levels of TNF-alpha, measured by a bioassay and immunoassay, and clinical manifestations of visceral leishmaniasis.

## CASUISTIC AND METHODS

### Patients

We evaluated nineteen patients with visceral leishmaniasis between August 1991 and July 1992, admitted to an urban Brazilian hospital (Giselda Trigueiro Hospital, city of Natal, Northeast region, Brazil). Cases were diagnosed based on clinical manifestations, and confirmed by the finding of *Leishmania* parasites in bone marrow and/or spleen aspirates. After obtaining informed consent, each case underwent daily clinical evaluation and routine laboratory workup during hospitalization. All diagnosed patients were treated with

meglumine antimoniate (Glucantime<sup>®</sup>). Twenty four healthy medical students and house staff from the Giselda Trigueiro Hospital and from a University Hospital in São Paulo were used as controls for baseline TNF-alpha levels.

Blood samples for TNF-alpha determinations were collected in heparinized tubes on days 0, 3, 7, 14, 21, and in some patients on day 60 of antimonial therapy. After separation, plasma was maintained frozen at -20°C in aliquots of 200-300  $\mu$ l and thawed just before use. Further laboratory evaluation included a complete blood count and biochemical tests. The size of spleen and liver was systematically recorded.

### Assay for TNF-alpha

Cytotoxicity assay was performed using sensitive L-929 cells and human recombinant TNF-alpha (Genzyme-USA) as previously described<sup>1</sup>. Samples were tested in triplicate and in four dilutions. One unit was defined as the TNF-alpha amount able to induce 50% cell lysis. Sample dilution and concentration of human recombinant TNF-alpha (hu r TNF-alpha, Genzyme USA) corresponding to 50% cell lysis, was calculated by regression analysis. Values expressed in units/ml were then converted to pg/ml using a reference curve obtained with hu r TNF-alpha in each assay. All samples from the same patient were tested in the same assay. One unit of TNF-alpha (50% cell lysis) corresponded to 14.4 pg/ml  $\pm$  5.3 pg/ml of hu r TNF-alpha. The specificity of the observed lysis was evaluated by adding rabbit polyclonal antibodies anti-human TNF-alpha to the samples (Genzyme-USA).

For enzyme-linked immunosorbent assay (ELISA) determination, a commercially available kit was used (Genzyme, USA), and the test was performed following manufacturer's instructions. Plasma samples and hu r TNF-alpha were tested in duplicate. Sensitivity of the test was 10 pg/ml.

### Statistical Analysis

Standard univariate statistical methods, including Student t-test, paired t-test, and repeated measures analysis of variance, were used to analyze the data. All analyses consisted of two-tailed tests, with an alpha level of 0.05 used to demonstrate statistical significance. Statistical programs 2D, 3D, 4F, and 2V of the BMDP Statistical Software Inc (University of California, 1992, Los Angeles, California, USA) were used.

## RESULTS

Low fever, hepatomegaly, splenomegaly, anemia, leucopenia, and hypoalbuminemia were commonly

**TABLE 1**  
Plasma levels of TNF-alpha, measured by ELISA, clinical and laboratory parameters in patients with Kala-azar, before, during and at end of antimonial therapy.

Parameter	Before (day 0)	During (day 14)	End (day 21)	n*	p value+ (day 0 versus day 21)
Spleen size (cm)	8.0 $\pm$ 3.9	5.3 $\pm$ 4.0	3.7 $\pm$ 3.1	18	<0.001
Hemoglobin (g/dl)	7.4 $\pm$ 2.0	9.1 $\pm$ 1.7	10 $\pm$ 1.6	18	0.002
White blood cell (cells/ml)	2863 $\pm$ 1562	3875 $\pm$ 1676	5188 $\pm$ 1573	18	0.004
TNF-alpha (pg/ml)	124.7 $\pm$ 93.3	36.8 $\pm$ 48.4	13.9 $\pm$ 25.1	14	0.001

\* = number of patients evaluated on days 0, 14 and 21.

+ = paired t-test

found in our cases (Table 1). Except for one patient who died on the 8th day of hospital admission due to bacterial pneumonia, all patients responded well to treatment with improvement in both clinical and laboratory parameters. Although some clinical and laboratory changes persisted at the end of treatment (21st day) (Table 1), these alterations were no longer present after 60 days of follow-up. Fifteen patients were available for evaluation at 6 and 12 months after treatment and all were asymptomatic.

Plasma levels of TNF-alpha were determined by ELISA in 14 patients and 11 controls. TNF-alpha was detected at day 0 in all but one patient (Figure 1), and in only two controls. Levels of circulating TNF-alpha declined significantly as clinical and laboratory abnor-

malities resolved (Table 1). Plasma levels of TNF-alpha and time of treatment were linearly associated ( $F=18.30$ ,  $p=0.003$ ) (Figure 1). The pretreatment level, 124.7 pg/ml  $\pm$  93.3 (mean  $\pm$  SD), was significantly higher than the level at day 21 of treatment, 13.9 pg/ml  $\pm$  25.1 (mean  $\pm$  SD) ( $p=0.001$ ).

TNF-alpha determinations by cytotoxicity assay were performed in all 19 patients studied. However, cytotoxicity assays were inconclusive in at least one sample of five patients (patients 3, 6, 7, 8 and 12). In those samples, cytotoxicity did not correlated with sample dilution, and cell lysis was not attributed to TNF-alpha. Therefore, cytotoxicity results of these five patients were not considered. In samples from day 0, TNF-alpha was detected in 5 of 14 patients, ranging

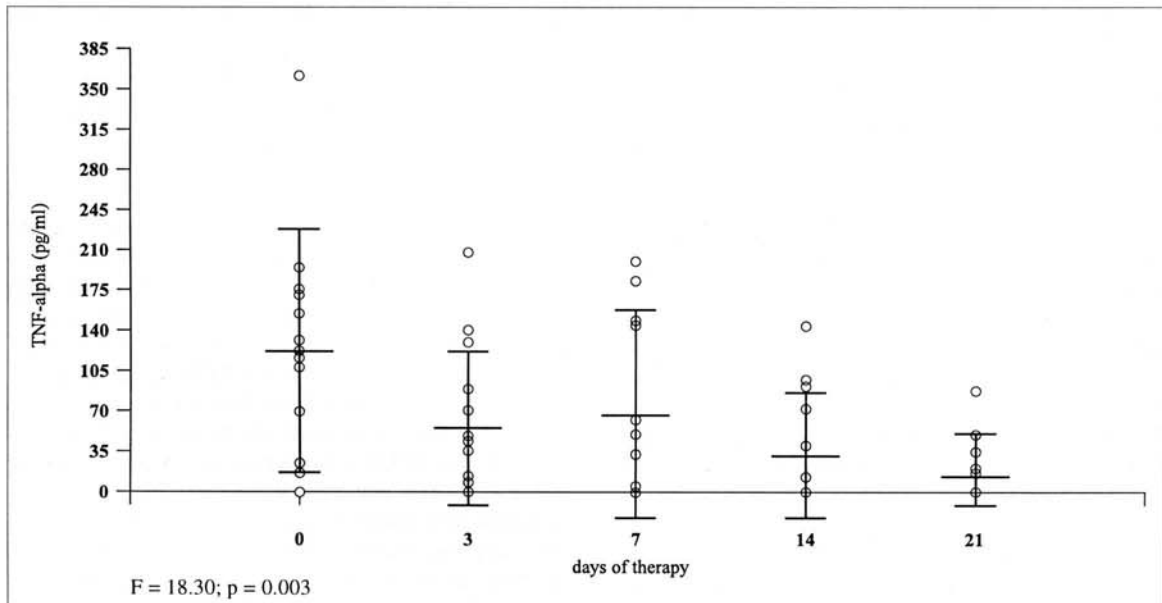


Fig. 1 - Association of plasma levels of TNF-alpha and time of therapy in patients (n=14) with visceral leishmaniasis.

TABLE 2

Plasma levels of TNF- $\alpha$  (pg/ml), measured by cytotoxicity assay, in patients with kala-azar before, during and at end of therapy. Values in parenthesis were obtained by ELISA.

Patients	Day 0	Day 3	Day 7	Day 14	Day 21
1	nd (nd)	nd (nd)	nd (nd)	nd (nd)	nd (nd)
2	nd	nd	nd	nd	nd
4	nd	nd	nd	nd	nd
5	nd	nd (40.9)	nd	nd	-
9	nd	nd	nd	56.6	nd
10	nd (103)	235.4 (13.5)	nd (nd)	nd (nd)	821.6 (nd)
11	261.8 (115.5)	nd (64.5)	512.3 (5.7)	nd (nd)	nd (nd)
13	453 (67.8)	nd (7.6)	93 (nd)	nd (nd)	nd (nd)
14	178.5 (193.9)	-	126.4 (134.6)	1050.7 (95.9)	426.6 (82.1)
15	nd	20.4	nd	nd	nd
16	nd (357)	-	nd (138.3)	nd (89.3)	882.7 (24.6)
17	883.5 (19.4)	nd (nd)	-	544 (nd)	nd (nd)
18	234	-	nd	nd	163.8
19	nd	88.4	nd	-	nd

nd = not detected; - = not done

from 178.5 pg/ml to 453.5 pg/ml. Levels of TNF-alpha measured by cytotoxicity assay did not decline as patients improved (Table 2). In some patients the levels of TNF-alpha were even higher in samples collected during therapy compared to initial samples. Seven patients had samples evaluated for TNF-alpha by ELISA and cytotoxicity. In contrast to results obtained with ELISA in these samples TNF-alpha detection by cytotoxicity did not follow a predictive course. Addition of polyclonal anti-TNF-alpha antibodies (Genzyme - USA) to samples obtained before, during and at the end of therapy dramatically reduced the cytotoxicity induced on L-929 cells (data not shown).

## DISCUSSION

Many clinical manifestations and laboratory alterations present in patients with Kala-azar are similar to the biological effects ascribed to TNF-alpha, and includes fever, anorexia, and weight loss<sup>8</sup>. We observed higher levels of TNF-alpha in patients with visceral leishmaniasis compared to healthy controls, as has been previously described<sup>20, 21</sup>. Further, in agreement with BARRAL et al.<sup>4</sup>, we demonstrated a negative correlation between levels of TNF-alpha and time of therapy and clinical recovery.

Plasma levels of TNF-alpha (measured by immunoreactivity) in patients with visceral leishmaniasis may therefore be indicative of disease activity and reduced levels observed during treatment could be considered a

marker of response to therapy. This is reinforced by demonstration of persistent high levels of TNF-alpha in patients with visceral leishmaniasis refractory to antimonial therapy<sup>4</sup>.

The role of TNF-alpha has been investigated in various infections and it has been associated with host-defense<sup>14,16</sup> or with increased severity of disease<sup>12,27</sup>. Although high levels of immunoreactive TNF-alpha are associated with disease activity in patients with kala-azar, the role of this mediator is still to be defined in this disease. In murine experimental models of tegumentar<sup>15</sup> and visceral leishmaniasis, TNF-alpha plays a protective role in host defense.

Interestingly, measurement of TNF-alpha by cytotoxicity assay showed discrepant results from those obtained by ELISA. Of concern, five patients were excluded from analysis because the results obtained by cytotoxicity were inconclusive in at least one sample. While TNF-alpha, measured by ELISA, decreased as patient improved (Figure 1 and Tables 1, 2), plasma levels of TNF-alpha assessed by the bioassay (cytotoxicity assay) did not follow a predictive course during therapy (Table 2). To rule out that cell lysis was due to contaminating bacteria, samples were cultured and were consistently negative. Further, filtering samples did not reduce cell lysis. However, this does not exclude the presence of bacterial products or another component of the sample which could induce cytotoxicity. Addition of an-

tibodies to TNF-alpha to some samples dramatically reduced, but not abolished, cytotoxicity of the samples. Therefore, lysis of L-929 cells observed may be not totally attributed to TNF-alpha activity.

Discrepancy between bioassay and immunoassay regarding detection of TNF-alpha has been described<sup>10,19</sup>. AUKRUST et al.<sup>2</sup> reported clearly different results on detection of serum levels of TNF-alpha in patients infected by the human immunodeficiency virus (HIV) measured by ELISA and cytotoxicity (100% and 27% positive, respectively), and attributed this discrepancy, at least in part, to the presence of high levels of TNF-alpha circulating receptor (TNF-R) in these patients. ENGELBERTS et al.<sup>9</sup> demonstrated that detection of TNF-alpha in presence of TNF-R was not altered by RIA, but was partially decreased by ELISA, and greatly impaired by cytotoxicity assay.

Concluding, our data shows association of high plasma levels of TNF-alpha (measured by ELISA) and activity of visceral leishmaniasis. A negative correlation between TNF-alpha and time on therapy and clinical recovery was observed. This correlation was not seen when TNF-alpha was measured by cytotoxicity. Such discrepancy may be due to the presence of factor(s) known to influence the release and activity of TNF-alpha, like other cytokines and circulating TNF-R.

## RESUMO

### Níveis plasmáticos do fator de necrose tumoral- $\alpha$ (TNF- $\alpha$ ) em pacientes com leishmaniose visceral (Calazar). Associação com atividade da doença e remissão clínica com terapia antimonial.

Avaliação de TNF- $\alpha$  em pacientes com calazar tem despertado grande interesse devido ao seu papel no sistema imunológico e à sua atividade caquetizante. O objetivo deste estudo foi examinar a associação entre os níveis plasmáticos de TNF- $\alpha$ , medidos através de sua imunorreatividade (ELISA) e bioatividade (ensaio citotóxico sobre as células L-929), e as manifestações clínicas da leishmaniose visceral. Amostras de 19 pacientes foram obtidas para determinação do TNF- $\alpha$  antes, durante e após a terapia antimonial, utilizando o ensaio de citotoxicidade (todos os pacientes) e o ELISA (14 pacientes). Resultados discrepantes entre os ensaios de citotoxicidade e o ELISA foram observados. Níveis circulantes de TNF- $\alpha$ , medidos pelo ELISA, foram mais altos nos pacientes que nos controles e declinaram significativamente com a melhora clínica e laboratorial. Níveis plasmáticos antes do tratamento (média = 124,7 pg/ml; DP = 93,3) foram mais elevados que ao final da terapêutica (13,9 pg/ml; DP = 25,1; p = 0,001). Por outro lado, níveis plasmáticos de TNF- $\alpha$ , avaliados pela

citotoxicidade, não seguiram um curso previsível durante a evolução. Esta discrepância pode ser devida à presença de fatores no plasma que podem influenciar a liberação e atividade do TNF- $\alpha$ . Ainda, a lise observada pode não ser totalmente atribuída ao TNF- $\alpha$ .

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