Tumor Necrosis Factor-α in Human American Tegumentary Leishmaniasis

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Tumor necrosis factor-alpha (TNF-α) is a cytokine produced by activated macrophages and other cells. In order to verify whether the serum levels of TNF-α in American tegumentary leishmaniasis patients are associated with the process of cure or aggravation of the disease, 41 patients were studied: 26 cases of cutaneous leishmaniasis (CL) and 15 of mucocutaneous leishmaniasis (MCL). During active disease the serum levels of TNF-α of MCL patients were significantly higher than those of CL patients and control subjects (healthy individuals and cutaneous lesions from other etiologies). The MCL patients had serum titers of TNF-α significantly lower at the end of antimonial therapy than before therapy. After a six-month follow-up, the MCL patients had serum levels of TNF-α similar to those observed at the end of the therapy as well as to those of CL patients and control subjects. No significant variation in the serum levels of TNF-α was observed in CL patients throughout the study period (before, at the end of therapy and after a six-month follow-up). The possible relationship between the high TNF-α serum levels and severity of the disease is discussed.

Key words: TNF-α - cytokine - tegumentary leishmaniasis - *Leishmania braziliensis*

In Brazil, American tegumentary leishmaniasis (ATL) is mainly caused by the protozoan parasite *Leishmania braziliensis*. Infection occurs by parasitization of phagocytic cells at the site where the infected Phlebotominae bites the host when taking bloodmeal. The most frequent clinical form of the disease, cutaneous leishmaniasis (CL), is characterized by single or multiple skin ulcers that may heal spontaneously or after therapy. However, a minority of patients develop a severe chronic form of the disease named mucocutaneous leishmaniasis (MCL) with secondary metastatic lesions in mucosae of the mouth and nose, that may result in severe destruction of the face. Moreover, MCL is frequently resistant to therapy.

Tumor necrosis factor alpha (TNF-α) is a cytokine produced mainly by activated macrophages but also by T lymphocytes, natural killer cells, and other cell types (Vassalli et al. 1992). It has a broad spectrum of biological functions on many different target cells, including cytotoxicity, inflammatory mediation, tissue remodeling and host defense against microbes (Beutler & Cerami 1988, Camussi et al. 1991). The two biological active forms of TNF-α, a soluble molecule (sTNF-α) and a membrane-associated protein (mTNF-α), seem to play different roles in the pathogenesis of several diseases, including experimental leishmaniasis (Kriegler et al. 1988, Birkland et al. 1992).

In experimental leishmaniasis TNF-α appears to play an important role in host defense (Titus et al. 1989, Liew et al. 1990a, b, 1991). On the other hand, in humans, higher titers of serum TNF-α do not appear to be associated with immune protection against *Leishmania* infection (Pisa et al. 1990) and also have been implicated in aggravation of many infectious diseases (Grau et al. 1989, Sarno et al. 1991, Barnes et al. 1992). In sera from patients with visceral leishmaniasis and diffuse cutaneous leishmaniasis relatively high titers of TNF-α have been observed (Pisa et al. 1990, Barral-Neto et al. 1991). The purpose of this study was to compare the serum levels of TNF-α in ATL patients with different clinical forms, namely CL and MCL, throughout their clinical evolution, from active disease to cure.

MATERIALS AND METHODS

Patients - Forty-one patients with ATL were studied. The following criteria were used for diagnosis: (a) Clinical and epidemiological data compatible with ATL; (b) Positive Montenegro skin test (MST) - delayed-type hypersensitivity (DTH)

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to leishmanial antigens. The test was considered positive when a enduration higher than 5 mm in diameter was observed after 48 hr; (c) IgG and/or IgM *Leishmania*-reactive antibodies, detected by indirect immunofluorescence in the serum. Titres were considered positive when fluorescence was observed at a 1:45 serum dilution or higher; (d) Detection of *Leishmania* in lesions either by microscopic examination of histological sections from biopsy samples or by culture in McNeal, Novy and Nicolle (NNN) medium (Nicolle 1908).

All patients were treated with antimonial (Glucantime®, Rhodia, São Paulo, SP, Brazil; three 10-day courses of N-methylglucamine at a dose of 15-20 mg Sb^{5+} daily i.m.). After a six-month follow-up the patients were considered cured if their lesions had completely healed.

Patients were evaluated before therapy (twenty CL patients and eleven MCL patients), at the end of therapy (fourteen CL patients and nine MCL patients) and after a six-month follow-up (thirteen CL patients and seven MCL patients). Some patients were evaluated in all periods of the study. As controls, five patients with cutaneous lesions from other etiologies and ten healthy individuals were also studied.

Serum samples - The sera were collected in sterile vacutainer blood collection tubes (Becton, Dickinson and Company, Rutherford, NJ, USA) before therapy, at the end of therapy and after a six-month follow-up period. Serum samples were immediately frozen at - 70°C and stored for a maximum of twelve months. No serum sample was frozen and thawed more than once.

TNF-α assay - Levels of TNF-α were determined by testing the sera using a solid phase enzyme-immunoassay employing the multiple antibody sandwich principle, as described in the kit specifications (Factor-Test™h TNF-α ELISA Test Kit, Genzyme, Cambridge, MA, USA). Briefly, human recombinant TNF-α, used as a standard in this assay, was serially diluted from 800 to 12 pg/ml. A 96-well microtiter plate was coated with mouse monoclonal antibody specific for human TNF-α and incubated overnight. Standard amounts of human recombinant TNF-α and serum samples were added in duplicate. Afterwards, the second antibody (rabbit anti-human TNF-α polyclonal antibody) and the third antibody (biotin-conjugated goat anti-rabbit IgG) were sequentially applied. Streptavidin-conjugated peroxidase was distributed into each well and a substrate reagent was added in order to obtain color reaction. The absorbance was measured at 492 nm in Multiskan Plus MK II spectrophotometer (Titertek®, Flow Laboratories, McLean, Virginia, USA). The results were expressed in pg/ml.

Statistical analysis -The Mann-Whitney two tail U test and Spearman correlation test were utilized.

RESULTS

Clinical findings - All patients had active leishmanial lesions at the beginning of the study. The mean age was 36.5±13.7 years for CL patients and 49.8±8.7 years for MCL patients. The mean period of illness was 2.4±1.8 months and 15.4±30.3 months, respectively for CL and MCL patients. With regard to the number of lesions in CL patients, 54% had a single lesion, 38% had two to ten lesions and 8% had more than ten lesions. All the MCL patients displayed lesions on the mucous membranes of the mouth and nose. All patients came from endemic areas of Rio de Janeiro, Brazil, where the only species of *Leishmania* that has been found infecting humans and dogs is *L. braziliensis* (Grimaldi Jr et al. 1991). The MST was positive in 88.4% of CL patients (mean = 10.3±3.5 mm in diameter) and 93.3% of MCL patients (mean = 16.5±8.1 mm in diameter). The diagnosis of leishmaniasis was established based in at least two of the criteria mentioned in the material and methods. At the end of therapy the lesions of all CL patients had healed while the lesions of MCL patients still displayed mild signs of activity. After the six-month follow-up all patients were considered clinically healed.

Serum levels of TNF-α - Sera from the patients with CL and MCL, as well as individuals with cutaneous lesions from other etiologies and from the healthy control subjects were tested for TNF-α. Before therapy, MCL patients displayed higher levels of TNF-α (mean = 231.4±76.3 pg/ml) than CL patients (mean = 43.5±8.2 pg/ml) ($p = 0.0004$). The latter had levels of TNF-α similar to those from patients with other cutaneous diseases (mean = 30.6±9.3 pg/ml) and healthy individuals (mean = 39.2±7.7 pg/ml) (Fig. 1). No significant correlation was observed between time evolution of the lesions and TNF-α serum levels in the leishmaniasis patients.

The mean levels of TNF-α in the sera from CL patients studied before therapy (twenty cases), at the end of therapy (fourteen cases) and after a six-month follow-up (thirteen cases) were respectively 43.5±8.2 pg/ml, 32.4±10.1 pg/ml and 37.4± 8.3 pg/ml. No significant difference among the levels of TNF-α of these three groups of CL patients (Fig. 2) was observed.

However, the serum levels of TNF-α in eleven MCL patients studied before therapy (mean = 231.4±76.3 pg/ml) were higher ($p < 0.05$) than those observed in nine MCL patients at the end of therapy (mean = 77.4±33.3 pg/ml) and in seven

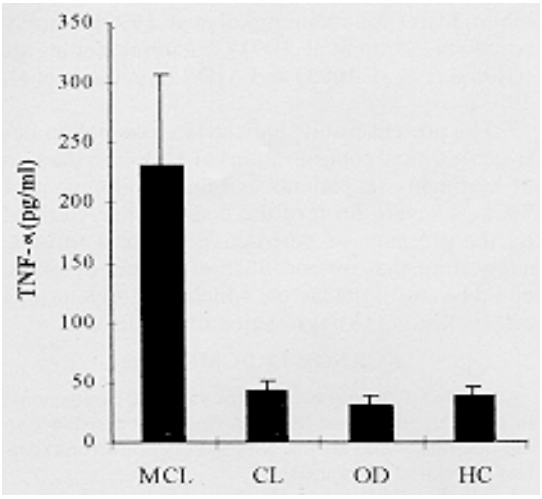

Fig 1: TNF-α levels (mean ± SE) in sera from twenty cutaneous leishmaniasis patients (CL) and eleven mucocutaneous leishmaniasis patients (MCL) before therapy, as well as patients with cutaneous diseases from other etiologies (OD) and healthy controls (HC).

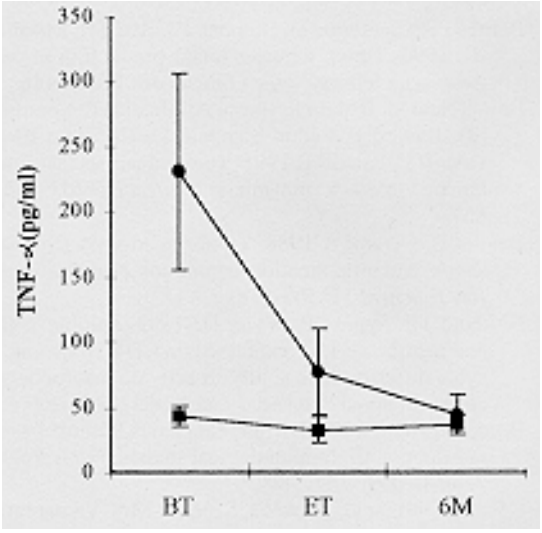

Fig 2: TNF-α levels (mean ± SE) in sera from patients with cutaneous leishmaniasis (CL) (■) and mucocutaneous leishmaniasis (●) tested in three occasions: before therapy (BT) (twenty CL patients and eleven MCL patients), at the end of therapy (ET) (fourteen CL patients and nine MCL patients) and after a six months follow-up (6M) (thirteen CL patients and seven MCL patients).

MCL patients after a six-month follow-up (mean = 45±15.5 pg/ml) (Fig. 2). The results of the latter two groups were not different from those of the CL patients (at any time of study) and control subjects (patients with cutaneous diseases from other etiologies and healthy individuals).

DISCUSSION

The active form of TNF-α is a trimer molecule which can be preserved at -70°C (Camussi et al. 1991, Thavasu et al. 1992). Under other conditions it is cleaved in monomer molecules that can also be measured, resulting in false results that are much higher than the actual levels. There is also a possibility of ex vivo induction of cytokines, including TNF-α, during the processing of blood samples (Leroux-Roels et al. 1988). For these reasons it is clear that a critical point for the measurement of the serum levels of circulating cytokines has been the establishment of optimal conditions for blood collection, processing and storage of the sample (Thavasu et al. 1992). In this study the samples were collected following these criteria, which suggests that the values found represent the actual levels of serum TNF-α.

The present results show that patients with active MCL, a severe clinical form of ATL, had levels of serum TNF-α significantly higher than CL patients (either active or healed). Clinically cured MCL patients had TNF-α levels significantly lower than those observed during active disease and similar to those of patients with CL and control subjects. The levels of TNF-α among CL patients did not show significant variations throughout the study period, which is in accordance with results reported by other authors (Pisa et al. 1990, Barral-Neto et al. 1991), although different parasite species and different human populations had been studied.

In human leishmaniasis high levels of TNF-α have also been associated with severe clinical forms such as active visceral leishmaniasis (Barral-Neto et al. 1991) and diffuse cutaneous leishmaniasis (Pisa et al. 1990), both characterized by a failure of the *Leishmania*-specific T cell-mediated immune responses (Bryceson 1970, Carvalho et al. 1988). In those cases, the observed high titers of TNF-α in the absence of an efficient T cell-mediated immune response was apparently not beneficial for the patient (Pisa et al. 1990). On the other hand, in the majority of MCL patients there is an exacerbation of the immune responses (Coutinho et al. 1987) with relatively higher frequencies of *L. braziliensis*-reactive T cells in the lesions (Conceição-Silva et al. 1990). In such cases the T cell-mediated immune responses appear to be associated with the process of aggravation of the lesions. It is possible that the higher levels of soluble TNF-α in the sera of MCL patients could be involved with pathogenic mechanisms. However, patients with CL, a clinical form characterized by the development of a well modulated T cell-mediated immune response, have TNF-α levels in their

sera similar to healthy subjects. It has also been demonstrated that the production of TNF-α by human T lymphocytes can be induced by the activation of molecules such as CD3 (Sung et al. 1988).

Studies of *in situ* production of TNF-α in human American tegumentary leishmaniasis. (Cáceres-Dittmar et al. 1993, Pirmez et al. 1993) have not shown significant differences in the TNF-α mRNA expression between MCL and CL lesions. However, the detection of mRNA may not necessarily correspond to the protein production *in situ* and/or seric levels, and also it did not discriminate between the two biological forms of TNF-α (soluble and membrane-associated) that appear to play different roles in the immunopatho-genesis of experimental leishmaniasis (Birkland et al. 1992). The soluble form having deleterious effects and the membrane-associated form with beneficial effects.

Studies on experimental murine leishmaniasis have indicated that TNF plays an important role in the immune protection against the disease. Local treatment of the leishmanial lesions with TNF significantly reduced their development (Liew et al. 1990b) not only by reducing the pathological damage but also inhibiting parasite replication. The last effect may be due to its ability to induce macrophage leishmanicidal activity mediated by nitric oxide (Liew et al. 1990a). Lymph node cells (LNC) from genetically resistant mice infected with *L. major* are able to produce higher levels of TNF than those from susceptible mice (Titus et al. 1989), although it was not clear which cells (macrophages or T lymphocytes) were the main producers of the cytokine. The production of TNF-α can be associated with unspecific tissue reactions or related to the presence of *Leishmania*-specific $CD4^+$ T cells able to produce and express mTNF on their cell surfaces leading to a increased ability to activate antileishmanial mechanisms of macrophages from resistant mouse strains (Birkland et al. 1992).

In leishmaniasis the host-protective effect of TNF-α that prevents lesion development could be associated with the presence of the mTNF-α expressed on macrophages or lymphocytes in the lesions. In CL patients an optimal production of mTNF-α in association with other cytokines could induce mechanisms involved in the healing of the lesions. On the other hand, an inappropriate release of soluble TNF-α could have deleterious effects, as suggested by the present results concerning MCL patients. High levels of TNF-α have also been associated with the pathogenesis of several damage conditions such as in *Plasmodium falciparum* malaria (Grau et al. 1989), streptococcal toxic shock syndrome (Hackett & Stevens 1992), meningococcemia (Waage et al. 1987), septicemic melioidosis (Suputtamongkol et al. 1992), leprosy reactions (Sarno et al. 1991), bacterial meningitis (Glimaker et al. 1993) and AIDS (Ayehunie et al. 1993).

The present results indicate an association between elevated concentrations of TNF-α in the sera of leishmaniasis patients and the development of MCL, a severe form of the disease characterized by the presence of destructive mucosal lesions, suggesting that overproduction of this cytokine could be one of the factors which have deleterious effects leading to aggravation of the disease.

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