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Original article

The immune response in patients with cutaneous leishmaniasis and the influence of zinc supplementation

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ABSTRACT

Cutaneous leishmaniasis triggers a varied immune response depending on parasite and host factors, which in turn can be influenced by nutrients. The resistance to the infection is associated with the Th1 type of cytokine production. The Th1 type can be reduced as a consequence of zinc deficiency, which may increase the risk for chronicity of the infection. Using in vitro and ex vivo models, we studied the influence of zinc supplementation on the immune response in patients with cutaneous leishmaniasis treated with antimony and the data were also compared to those of matched controls. Twenty-nine patients with cutaneous leishmaniasis ($n = 14$ in zinc-supplemented group [45 mg/day] and $n = 15$ in placebo group) were treated by intramuscular injections of antimony for 20 days and took supplements for 60 days. Immunoglobulins in plasma and cell proliferation, IFN-γ production and CD markers of isolated peripheral blood mononuclear cells (PBMC) were measured. It was found that the cellular immune response of the patients maintained its activity as assessed by the ability of the PBMC to proliferate and produce IFN-γ in response to concanavalin A. Moreover, there was no difference in these variables between the zinc-supplemented and placebo groups after 60 days. The addition of zinc sulphate in vitro to PBMC reduced the IFN-γ production in the placebo group only. It is concluded that the cellular immune response of the cutaneous leishmaniasis patients remained active during treatment by antimony when compared to that of controls. It was not possible to document an additional effect of zinc supplementation for 60 days on the immune response.

1. Introduction

Cutaneous leishmaniasis triggers a complex and varied immune response depending on the influence of host factors and the parasite species [1,2]. Some of the factors controlling the immune response in turn are also affected by dietary factors [3]. The host resistance to leishmaniasis in an animal model was associated with a Th1 type response characterized by increased production of interferon-gamma (IFN-γ) and IL-2 [1,2,4]. Data obtained in human volunteers are contradictory because some authors have shown that more IFN-γ was produced by CD8+ T cells than by CD4+ T cells [5] and others reported that IFN-γ and IL-4 was produced by CD4+ T cells and the suppression of these cells inhibited their production [6]. Other evidence not related to leishmania infection indicates that production of Th1 cytokines can be reduced as a consequence of zinc deficiency, which induces a shift of the balance Th1/Th2 towards Th2 [7]. This can in turn cause immune dysfunction, secondary infections and chronicity of pre-existing infections [8,9]. Data obtained in humans have also indicated that the CD4+/CD8+ ratio is decreased during zinc deficiency [8] and another study showed a higher number of CD8+ lymphocytes in

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zinc-deficient subjects [10]. The available data on the effect of zinc addition on the in vitro T cell proliferation in response to mitogen stimulation are contradictory and both an increase and an inhibition of the proliferation have been reported [8,11].

The effect of zinc on the immune response in general has been much studied, but there are few data available regarding the modulation of immune response by zinc during leishmaniasis infection. In a related disease, namely an experimental infection with *Trypanosoma cruzy*, enhanced IFN-γ concentrations and reduction in parasitemia levels were demonstrated in infected animals supplemented with zinc during 14 days [12,13]. To gain more knowledge on these topics, we used an ex vivo model to compare immune status in patients with cutaneous leishmaniasis and matched controls and to evaluate the influence of zinc supplementation on the immune response in the patients.

2. Materials and methods

2.1. Patients

Thirty-four out of 87 patients met the inclusion criteria and were invited to participate in the study as described elsewhere [14]. They resided in the tropical area of Cochabamba province, Bolivia (16°39́–17°25́ South latitude; 64°14́–65°44́ West longitude). All patients had active cutaneous leishmaniasis as diagnosed by findings of amastigote or promastigote parasites by microscopy of stained smears of scrapings of border lesions and/or by isolation in culture as performed at Villa Tunari Hospital [15]. They also met the additional selection criteria, namely age 15–50 years and no previous leishmaniasis episodes. Exclusion criteria were mucosal or mucocutaneous leishmaniasis, presence of more than two cutaneous ulcers, use of nutritional supplements, pregnancy, lactation, HIV infection, diabetes mellitus, chronic renal failure or liver disease. All patients completed a health questionnaire prior to entering the study and signed a consent form for inclusion into the study.

2.2. Control subjects

The controls were age- and gender-matched subjects without any clinical signs of previous cutaneous leishmaniasis and also residents in the same area as the corresponding patients. Exclusion criteria were pregnancy, lactation, diabetes mellitus, chronic renal failure or liver disease or use of regular medication or nutritional supplements. All controls completed a health questionnaire prior to entering the study and signed a consent form.

2.3. Study design

Patients were randomly allocated by a lottery system to receive daily zinc or placebo coded capsules for 60 days. Each zinc capsule contained 315 mg of zinc gluconate (corresponding to 45 mg zinc) and each placebo capsule contained 315 mg of cornstarch, both specially prepared for our study by a company (Farmacia Artesanal, Cochabamba, Bolivia). All patients received also for 20 days the conventional treatment for leishmaniasis by daily intramuscular injections of pentavalent antimony (meglumine antimoniate, Glucantime®, Sanofi Aventis Farmacêutica Ltda, São Paulo, Brazil), 20 mg Sb/kg/day. Control subjects were not given any drugs or zinc or placebo capsules. For patients, blood was sampled three times in the study, at time zero (T0) before starting anti-leishmaniasis therapy and supplementation, at the end of anti-leishmaniasis therapy (20 days, T1) for renal and hepatic function evaluation only (the results are presented elsewhere [14]) and at the end of the supplementation period (60 days, T2). For controls, blood was sampled at time zero only.

2.4. Collection of blood samples

Blood was collected by venipuncture into heparinized tubes (after 12 h fasting and 30 min of relaxing) at Villa Tunari Hospital. Plasma for clinical chemistry analyses was obtained by centrifugation within 30 min of sampling, aliquoted and stored at −80 °C until analysis. Blood for isolation of peripheral blood mononuclear cells (PBMCs) was centrifuged approx. 6 hours after sampling after transport from Villa Tunari to Cochabamba, and the cell pellet was used for the cell culture in the IIBISMED laboratory as described below.

2.5. Measurement of immunoglobulins, C-reactive protein and specific anti-leishmania IgG

The measurement of plasma immunoglobulin concentrations (IgA, IgG and IgM) and C-reactive protein (CRP) was performed at the clinical chemistry laboratory of Skåne University Hospital, Lund, Sweden. The samples were transported on dry ice from Bolivia to the laboratory. The analytical methods involved the addition of antibodies directed to the different type of immunoglobulins and CRP and the resulting agglutinates were measured by turbidimetry on a Cobas instrument using accredited methods.

The measurement of specific plasma anti-leishmania IgG was performed following the procedure of the Immunology laboratory of the Medicine Faculty, Universidad Mayor de San Simón. Briefly, the plasma samples were diluted 1:40 with buffered saline solution and applied on slides previously coated with leishmania parasites as antigen. After 30 min of incubation at 37 °C anti-human IgG labelled with fluorescein isothiocyanate was added and incubation was continued for 30 min. The slides were evaluated by immunofluorescence microscopy. The results were expressed as positive or negative according with the intensity of fluorescence compared with the positive and negative controls.

2.6. Measurement of proliferation of peripheral blood mononuclear cells

After removal of plasma by centrifugation (5000 g for 10 min) twice the volume of phosphate-buffered saline (PBS) was added to the cell pellet. The diluted cell suspension was layered onto histopaque (density 1.077; ratio of diluted cell suspension to histopaque 2:1) and centrifuged for 30 min at 3000 g. The PBMCs obtained were washed twice with RPMI-1640 containing penicillin/streptomycin (1:100) and finally re-suspended in the same medium. The PBMC suspension was adjusted to a final concentration of 1×10^6 cells/ml after performing a manual cell counting (dilution 1:100 with Türk solution). The viability was determined by cell counting in a Neubauer hemocytometer after dilution 1 + 1 with Trypan blue solution and the cell viability expressed as mean (S.D.) was 84% (12%).

PBMCs (1×10^6 cells) were cultured in RPMI-1640 medium, fetal bovine serum (FBS) and antibiotics. For stimulation of cells, concanavalin A (Con A, final concentration 5 μg/ml), or a mixture of Con A plus Zn (1 μmol/L $ZnSO_4$) were added. Con A was selected based on its property of act as a polyclonal stimulus of T cells. The final volume of the culture was 300 μl/well and all cultures were performed in triplicate. The proliferation was measured after 6 days of incubation as total DNA content as determined by propidium iodide staining [16]. Briefly, the cells were permeabilized for 30 min with 500 μl of 100% ethanol and incubated in the dark for 30 min with 500 μl of propidium iodide (10 μg/ml) in PBS. The fluorescence (excitation, 535 nm;