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Original Contribution

Excreted *Leishmania peruviana* and *Leishmania amazonensis* iron–superoxide dismutase purification: Specific antibody detection in Colombian patients with cutaneous leishmaniasis

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ABSTRACT

Leishmania sp. survival in the vertebrate host depends on the host macrophage immune response as well as on the parasite's defense against free radicals. Iron–superoxide dismutase (Fe-SOD) is a key antioxidant enzyme that contributes to radical superoxide dismutation, preventing the disease from surging and propagating itself. *Leishmania* sp. has various Fe-SOD isoforms, one of which (Fe-SODe) is excreted into the medium and, being highly immunogenic, can be considered a very good molecular marker. In this work, we purified the Fe-SOD enzymes excreted by *L. peruviana* and *L. amazonensis* and studied them as antigens in serodiagnosis. We used ELISA and Western blot techniques to test 51 human cutaneous leishmaniasis sera from Colombia. All 51 patients presented with dermal injuries caused by unknown *Leishmania* species. The results observed with the purified proteins were compared with those obtained when total soluble lysate and unpurified Fe-SODe were used as the antigen fraction. Thus, we conclude that the purified enzymes are more sensitive and specific than their unpurified counterparts and that there is no cross-reactivity between them.

Leishmaniasis constitutes a group of infectious diseases caused by protozoan parasites of the genus *Leishmania,* of which more than 20 species are pathogenic for humans.

This disease presents various clinical manifestations, which can be classified into three large groups: visceral, cutaneous, and mucocutaneous. These parasites are distributed worldwide and in 2001 the World Health Organization (WHO) in their Tropical Disease Research Programme recognized leishmaniasis as one of the six most important tropical diseases, together with malaria, schistosomiasis, filariasis, leprosy, and trypanosomiasis. At the present time, leishmaniasis is endemic in 98 countries and territories, in which 12 million people are infected and more than 250 million people have been estimated to be at risk of contracting the disease [1].

Leishmania spp. parasites are digenetic, and their proliferation in the vertebrate host occurs in the mononuclear phagocyte system, which is part of the immune system.

Although *Leishmania* spp. can invade various cells, they selectively target macrophages, in which the parasite replicates itself and starts the infection. Macrophages are then activated and are known as "effector cells" [2]. After that, various cellular processes such as phagolysosomal degradation, oxidative-burst generation, and nitric oxide (NO) production begin, leading to phagocytosis and destruction of the unwanted host [2].

Promastigotes released into the vertebrate host's bloodstream during the bite of an infected sand fly are phagocytosed by macrophages. Similarly, amastigotes released after rupture of their host macrophage are quickly phagocytosed by new macrophages. The fate of the *Leishmania* amastigote forms inside the macrophages is determined by the activation state of the latter [3]. Macrophages can be activated in two ways, the classical (M1), by Th1 lymphocytes, and an alternative one (M2), by Th2 lymphocytes [4]. It has been shown that *Leishmania* spp. have an enhanced uptake of L-arginine, because they lack the ability to synthesize it de novo. *Leishmania major* is the prototypical model of Th1 and Th2 responses. It has been proposed that Th2 activation by *L. major* involves an increase in the production of polyamine, which affects the thiol/sulfide redox balance, thereby increasing the antioxidant defenses [4].

The macrophage processes acting against *Leishmania* sp. may be oxygen-dependent or oxygen-independent mechanisms. Macrophages activated by Th1 lymphocytes excrete interleukin-12 (IL-12), activating the natural killer cells (NKs). NKs induce interferon-γ (IFN-γ) production and its secretion, this being stimulated by IL-2. Macrophages, induced by IFN-γ, release tumor necrosis factor-α, which acts synergistically with IL-12 and IFN-γ,

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leading to NO production, which starts from the L-arginine pathway via inducible nitric oxide synthase [5]. The end-products are NO and the superoxide ion ($O_2^{\bullet -}$). These two products together produce peroxynitrite ($ONOO^-$), resulting in cell death [4,6,7]. It is not yet clear whether the decreased virulence is caused by reduced L-arginine levels in the macrophages, with a consequent reduction of NO and ROS (reactive oxygen species) concentrations, or by a rise in polyamine levels inside the parasite. Polyamines are tools used in the synthesis of enzymes containing thiol groups, which are involved in the parasite's antioxidant defense, and favor the propagation of *Leishmania* inside the host [4]. A massive presence of ROS and reactive nitrogen species can lead to nucleotide deamination, protein tyrosine nitration, and irreversible inhibition of metalloenzymes [8]. $O_2^{\bullet -}$ is produced by Nox enzymes present in macrophages during the phagy stage of infection and also by *Leishmania* sp. promastigotes, and it is responsible for most intracellular ROS [9]. The high toxicity of $O_2^{\bullet -}$ and H_2O_2 is enhanced by the formation of the hydroxyl radical ($HO^{\bullet}$), which is produced by Fenton's reaction [10].

The iron–superoxide dismutase (Fe-SOD) enzyme protects the parasite against radical $O_2^{\bullet -}$ by dismuting $O_2^{\bullet -}$ to H_2O_2 and O_2 during phagocytoses as well as during the intracellular state in both amastigote and promastigote forms. In turn, hydrogen peroxide is reduced to H_2O by the antioxidant defense of *Leishmania* sp.: trypanothione reductase, trypanoredoxin, peroxiredoxin, and ascorbate peroxidase [2,11,12], all of which are thiol enzymes involved in redox balance and responsible for parasite survival and propagation of the disease.

Fe-SOD has been identified in a few protozoa, such as *Plasmodium* spp. [13], *Acanthamoeba castellani* [14], *Entamoeba histolytica* [15], *Toxoplasma gondii* [16], *Perkinsus marinus* [17], and *Trichomonas vaginalis* [18]. Different isoforms of Fe-SOD have also been identified in the trypanosomatids family. *Chritidia fasciculata* presents three isoforms [19], *Phytomonas* sp. two [20], and *Trypansoma cruzi* [21] and *T. brucei* [22] both present four. In *Leishmania* spp. the number of isoforms depends on the species: *L. infantum* has three isoforms [23], *L. peruviana* four, whereas *L. amazonensis* and *L. peruviana* both present five isoforms [24,25].

It has been demonstrated that the excreted isoform of SOD is the most relevant as it plays an important role in the pathogenesis of pulmonary, neuronal, and cardiovascular diseases [26,27].

The excreted isoform has been identified in *Collinectes sapidus* [28], *Burghia pahangi* [29], *Oncocherca volvulus* [30], and *Caenorhabditis elegans* [31] and has been proven to elicit the immune response.

Marín and co-workers [32] described for the first time in the year 2006 the immunogenicity of Fe-SOD excreted by *Phytomonas* sp., which presented a molecular weight (MW) of approximately 22 kDa and an isoelectric point (p*I*) of about 3.6. Later, immunogenicity of the *T. cruzi* Fe-SODe was also shown. In this parasite the protein had a MW of about 25 kDa and a p*I* of approximately 3.8 [33].

Indeed, Fe-SODe has also been shown to be a very good molecular marker with high sensitivity toward various trypanosomatids, such as *Phytomonas* sp. [20,32], *T. cruzi* [21,33], and *Leishmania* spp. [34]. In recent years, our group has proposed the use of Fe-SODe excreted by *Leishmania* spp. as an antigen, taking advantage of its high immunogenicity and sensitivity in cutaneous, mucocutaneous, and visceral leishmaniasis diagnosis in humans and in other mammals, such as cats or dogs [34–37].

We proved recently that the Fe-SOD excreted by *L. infantum*, which was purified with the same method used in the present work, gave results of a higher sensitivity and specificity in canine leishmaniasis diagnosis than those obtained from the same enzyme without purification [38]. In the present work we purified the Fe-SOD excreted by *L. peruviana* and *L. amazonensis* (SODe-Lp and SODe-La, respectively) and used them in the diagnosis of human cutaneous leishmaniasis. We tested 51 sera taken from Colombian subjects; all the patients had cutaneous lesions produced by an unidentified *Leishmania* sp. when the blood sample was taken. The main aim of this work was to confirm the high sensitivity and specificity of this purified antigen and to prove its potential utility in human leishmaniasis diagnosis, with no cross-reactivity among different *Leishmania* species. The absence of cross-reactivity is critical, especially in countries where more than one endemic *Leishmania* species present the same clinical manifestations.

Materials and methods

Parasite culture

Promastigotes of *L. peruviana* (MHOM/PE/84/LC26) and *L. amazonensis* (MHOM/BR/73/M1845) were grown in tissue-culture flasks and an axenic medium trypanosomes liquid (MTL) medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 26 °C until reaching a population of approximately 1×10^7 parasites/ml. Cells were collected at the logarithmic growth phase by centrifugation (1500 *g* for 10 min at room temperature). The cell pellet (0.5–0.6 g wet wt/ml) was suspended in 25 ml of MTL medium that had not been enriched with FBS and cultured at 26 °C for 24 h.

Antigen preparation: extraction and purification of Fe-SODe

After 24 h, the promastigote culture was centrifuged (1500 *g* for 10 min) and the supernatant filtered (Minisart, Φ 20 μm). The filtered supernatant was subjected to ice-cold ammonium sulfate precipitation at 35% salt concentration. After centrifugation, the resultant supernatant was treated with 85% ice-cold ammonium sulfate and the second precipitate collected. The resulting precipitate was finally dissolved in 2.5 ml of distilled water and desalted by chromatography in a Sephadex G-25 column (GE Healthcare Life Sciences, PD 10 column) previously equilibrated with 25 ml of distilled water, bringing it to a final volume of 3.5 ml (fractions P85e-La and P85e-Lp, respectively, or SODe-np-La and SODe-np-Lp) [20]. Degradation due to the activity of protease present in the sample was minimized by adding 25 μl of protease inhibitor to fraction P85e (CompleteMini, Roche). Fraction P85e was introduced into a QAE-Sephadex A-50 column (Sigma Immunochemicals, St. Louis, MO, USA), 30 × 2-cm Φ, equilibrated with buffer 1 (20 mM potassium phosphate, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA)). The adsorbed proteins were eluted with a linear gradient of KCl (0–0.6 M). Fractions with a total volume of 2.5 ml were re-collected. Fractions exhibiting specific SOD activity were pooled (peak Q1e-La and peak Q1-Lp, respectively) and concentrated by ultrafiltration in Microcon filter tubes (Amicon) at 11,200 *g* for 30 min. The peak Q1e was introduced into a Sephadex G-100 (Sigma Immunochemicals) molecular sieve chromatography column (75 × 1.6-cm Φ) equilibrated with buffer 1 and eluted with 200 ml of the same buffer. The eluted fractions (4.5 ml each fraction) that showed SOD activity were again collected (peak SODe-La and peak SODe-Lp, respectively), concentrated (to 2 mg/ml), and used for the assays described below.

Protein determination

The protein content of the fractions P85e and the peaks Q1 and SODe, and all the fractions resulting from the elution of the two columns, were quantified using the Sigma Bradford test, which uses bovine serum albumin (BSA) as a standard (no traceability was certified for the BSA standard) [39].

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