Sporothrix schenckii Lipid Inhibits Macrophage Phagocytosis: Involvement of Nitric Oxide and Tumour Necrosis Factor-α

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Abstract

The role of cell-wall compounds in the immune response to sporotrichosis is unknown. The effect of cell-wall compounds and exoantigen obtained from *Sporothrix schenckii* in macrophage/fungus interaction was analysed with respect to nitric oxide (NO) and tumour necrosis factor-α (TNF-α). The lipid compound of the cell wall plays an important role in the pathogenesis of this mycosis and was found to inhibit the phagocytic process and to induce high liberation of NO and TNF-α in macrophage cultures in the present study. This is a very interesting result because it is the first report about one compound of the fungus *S. schenckii* that presents this activity.

Introduction

Sporotrichosis is a chronic granulomatous mycosis caused by the dimorphic fungus *Sporothrix schenckii* [1–3]. In the past, the infections caused by *S. schenckii* were limited to the cutaneous form of the disease. Today, the occurrence of disseminated infections has been reported, especially amongst immunologically depressed patients [4–6]. Elimination of micro-organisms by macrophages depends both on phagocytosis and on the release of toxic agents, such as reactive oxygen and nitrogen intermediates. Nitric oxide (NO) is known to play an important role in the elimination of intracellular parasites, viruses and some bacteria like mycobacteria [7]. With increasing information about the regulation of immune reactions, it has become apparent that these responses are controlled, in part, by soluble mediators (cytokines) produced by macrophages. Some pro-inflammatory cytokines, notably interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) and the bacterial lipopolysaccharide (LPS), are potent agents stimulating the induction of inducible NO in the macrophages (iNOS, inducible NO synthase) [8]. The binding of non-opsonized micro-organisms to the macrophage membrane may be inhibited by carbohydrates present in the cell wall of these organisms. LPS sugar compounds inhibit the binding of *Salmonella typhimurium* to macrophages, suggesting the presence of membrane macrophage receptors that recognize a determinant of carbohydrate diversity contained in the wall of the micro-organism [9, 10].

The role of cell-wall compounds in the immune response to sporotrichosis is unknown. We have demonstrated the probable function of ergosterol peroxide isolated from yeast forms of *S. schenckii* in the virulence of this pathogenic fungus [11]. In the present study, we analysed the role of cell-wall compounds and exoantigen obtained from *S. schenckii* in the macrophage/fungus interaction with respect to NO and TNF-α.

Materials and methods

Reagents and media. Tissue culture medium RPMI-1640, fetal bovine serum, sulphanilamide, naphthylethylenediamine dihydrochloride, phosphoric acid (H_3PO_4), aminoguanidine hemisulphate salt (AG), sodium thioglycollate, NaCl, dextrose, phenol red, type II horseradish peroxidase, zymosan, actinomycin D, crystal violet, sodium dodecyl sulphate (SDS), recombinant TNF-α, rabbit anti-r TNF-α, mannose, glutaraldehyde, LPS

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(*Escherichia coli* 0111B) and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Complete tissue culture medium (CTCM) consisted of RPMI-1640 containing 5% (v/v) heat-inactivated fetal bovine serum, 100 U of penicillin, 100 μg of streptomycin and 5×10^{-2} M mercaptoethanol per ml.

Animals. Swiss mice weighing 18–25 g from the Animal House of the Faculty of Pharmaceutical Science of Araraquara were used in all experiments.

The fungus. S. schenckii, strain 1099-18, was kindly provided by Dr Celuta Sales Alviano, Institute of Microbiology, Federal University of Rio de Janeiro, RJ, Brazil. This strain was isolated from a human case of sporotrichosis at the Mycology Section of the Department of Dermatology, Columbia University, New York, NY, USA. The fungus was cultured at 37 °C for 8 days in brain heart infusion broth (DIFCO, Detroit, MI, USA) with constant shaking at 150 cycles/min.

Treatment of walls. The freeze-dried yeast cells were disrupted by six cycles of ultrasonic vibration at 200 W for 3 min. A high degree of disruption was always observed by phase-contrast microscopy. The walls were collected and washed with distilled water by centrifugation at $5000 \times g$ for 5 min. Washing was continued until phase-contrast microscopy indicated that the suspensions were free of undisrupted cells and cytoplasmic components. The freeze-dried wall samples were subjected to lipid extraction by soaking in chloroform/methanol (2 : 1, v/v), with stirring at room temperature for 2 h. The extract was separated by centrifugation at $5000 \times g$ for 5 min, and the insoluble residue was re-extracted three more times as described. The component was called lipid extract (LE) [12, 13].

Partial wall fractionation. This was performed by alkaline extraction, as described by Kanetsuma *et al.* [14, 15]. Briefly, walls were suspended in 1 N NaOH (10 mg/ml) and gently stirred at room temperature for 1 h. After centrifugation at $5000 \times g$ for 10 min, the supernatant was collected and the procedure repeated four times, combining all the supernatants. The alkali-insoluble sediment was washed with water until it reached pH 7.0 and then with ethanol, followed by acetone and diethyl ether. The resulting white powder was called alkali-insoluble fraction F1 (F1).

Exoantigen derivation. Fungus was cultured as described above, and the fungus culture was subjected to ultraviolet (UV) radiation for 1 h. This culture was maintained at 37 °C for 24 h and then re-subjected to UV radiation for 1 h, as described above. After this procedure, Merthiolate was added to the culture medium at 1/5000 concentration. The culture thus prepared was frozen at −20 °C for 48 h. Next, culture sterility was tested by the Sabouraud agar test, and the culture was filtered and concentrated 50–100 times in a concentrator (Amicon 8050, Amicon, Danvers, MA, USA). Protein measurement (3.0 mg/ml) was carried out by the method of Lowry *et al.* [16].

Peritoneal macrophages. Thioglycollate-elicited peritoneal exudate cells (PECs) were harvested from Swiss mice using 5.0 ml of sterile phosphate-buffered saline (PBS), pH 7.4. The cells were washed twice by centrifugation at $200 \times g$ for 5 min at 4 °C and resuspended in appropriate medium for each test.

Hydrogen peroxide measurement. PECs (2×10^6 cells/ml) were cultivated as described above and suspended in a solution containing 140 mmol NaCl, 10 mmol potassium phosphate buffer, pH 7.0, 5.5 mmol dextrose, 0.56 mmol phenol red and 0.01 mg/ml type II horseradish peroxidase. Next, 100 μl of this suspension was added to each of the wells of a 96-well flat-bottom tissue culture plate along with 50 μl of exoantigen (ExoAg) solution at 0.5, 1.5 or 3.0 mg/ml, F1 and LE solution at 1.0, 2.0 or 5.0 mg/ml or 50 μl of 5.0 mg/ml zymosan solution in phosphate buffer for the positive control wells. The cells were incubated for 1 h at 37 °C in a mixture of 95% air and 5% CO_2. The reaction was stopped with 10 μl of 4 N NaOH, and the samples were read at 620 nm against a blank containing phenol red solution and 4 N NaOH, the results being expressed as nanomoles of hydrogen peroxide (H_2O_2) per 2×10^5 cells as calibrated against solutions of known H_2O_2 concentration. At least four experiments were used for the test [17, 18].

NO measurement. The PECs were resuspended in CTCM at a concentration of 5×10^6 cells/ml, and 100 μl of this suspension was added to each well of a 96-well tissue culture dish along with 100 μl of ExoAg solution at 0.5, 1.5 or 3.0 mg/ml, F1 and LE solution at 1.0, 2.0 or 5.0 mg/ml or 100 μl of a 10.0 μg/ml (1.0 μg) *E. coli* 0111B LPS solution as positive control. The cells were incubated for 24 h before stimulation, and 50 μl aliquots of culture supernatant were mixed with 50 μl of Griess reagent (1% w/v sulphanylamide, 0.1% w/v naphthylethylenediamine and 3% H_3PO_4), incubated at room temperature for 10 min, and the colour reaction was determined at 540 nm with a Multiskan Ascent Enzyme-Linked Immunosorbent Assay (ELISA) reader (Labsystems, Helsinki, Finland) [19]. Supernatants from quadruplicate cultures were assayed in four experiments and reported as mean NO_2^- concentration ± standard error of the mean (SEM).

TNF-α production and assay. For TNF-α production, adherent PECs were stimulated with 50 μl of each solution: 10.0 μg/ml of LPS (*E. coli* 0111B) or 5.0 mg/ml of LE and F1 or 3.0 mg/ml of ExoAg. After 24 h, the supernatants were removed, filter-sterilized and stored at −80 °C until assayed. L929 mouse tumour cells were used to measure TNF-α levels in macrophage supernatants, as previously described [20]. Killing of L929 mouse tumour cells was as a measure of the cytotoxicity of soluble factor(s) present in the supernatants. Briefly, L929 cells in RPMI-1640 medium containing 5% (v/v)

fetal calf serum (Sigma) were seeded at 4×10^4 cells per well in 96-well microtitre plates (Corning, Inc, Corning, New York, NY, USA) and incubated overnight at 37 °C in an atmosphere of air/CO_2 (95 : 5, v/v). Serial 1 : 2 dilutions of the culture supernatants previously obtained were prepared in the above medium containing 1.0 μl of actinomycin D per ml (Sigma), and the cell-culture medium was replaced with 100 μl volumes of the dilutions in triplicate. The next day, cell survival was assessed by fixing and staining the cells with crystal violet (0.2% in 20% methanol), dissolving the stained cells with 0.1 ml 1% SDS (w/v) per well and reading the absorbance of each well at 490 nm with a Multiskan Ascent ELISA reader (Labsystems). TNF-α units were calculated using a standard curve obtained with recombinant TNF-α constructed for each test run. To confirm the presence of TNF-α in the culture supernatants, these preparations were previously incubated with rabbit anti-r TNF-α immune serum, and the test samples were added to L929.

Inhibition of phagocytosis. For the inhibition experiments, 100 μl of each ExoAg solution (0.5, 1.5 or 3.0 mg/ml) and F1 and LE (1.0, 2.0 and 5.0 mg/ml) solution in PBS, pH 7.4, was added to the slide-adhered macrophages from 1.5×10^6 PECs (100 μl). After 20 min of exposure, cells were gently washed, and 0.25 ml of a yeast suspension of $2.5 \times 10^7 - 5.0 \times 10^7$ cells per ml was added. Slides were incubated for 90 min at 37 °C and washed with PBS containing 0.2 M mannose to remove yeast cells adhered to the outer surface of macrophages as well as remove unbound yeasts. Cells were fixed with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 30–60 min and by brief heating. Cells were stained by the May–Grünwald–Giemsa method. An average of 200 macrophages were counted on several microscope fields to determine the percentage of macrophages phagocytosing at least one yeast cell (*P*) and the average number of yeasts in these macrophages (*F*). The phagocytic index (*I*) was determined by multiplying *P* and *F* [21]. Some experiments were performed with fungus treated with FITC before the phagocytosis assay [22].

Statistical analysis. Results are representative of three independent experiments and are presented as the mean ± SEM of quadruplicate observations ($n = 12$). Data were analysed statistically by the Student's *t*-test using the ORIGIN statistical software version 5.0.

Results

Reactive nitrogen and oxygen intermediates induced by *S. schenckii* cell derivatives

NO, together with oxygen radicals, contributes to the cytotoxic activity of phagocyte leucocytes (including macrophages) toward certain bacteria, protozoan parasites, fungi and viruses. Figure 1 shows that the H_2O_2 mediator was mainly released by the F1 fraction, whereas Fig. 2 demonstrates that LE released more NO when compared with other fractions (F1 and ExoAg). These results led us to consider the participation of distinct fractions in the immune response and to propose that the interaction of these reactive molecules would, especially, probably occur at the site of infection, contributing to the modulation of the immune response or to the cytotoxic effects. The cytotoxic effect of these LE concentrations tested on cultured macrophages was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described [23] with a 5–10% average reduction in stimulated versus control cell number at 24 h of incubation (data not shown).

Release of TNF-α

The interrelationship between cytokines and reactive nitrogen has been clearly established. Cytokines are important in the acute or chronic inflammatory response initiated by infection or trauma. In the present study, TNF-α was preferentially induced by LE (Fig. 3), as observed in NO determination. It is known that TNF-α is produced by LPS and plays a role in inflammation, and although TNF-α is not an inducer of iNOS, it is crucial for the synergistic

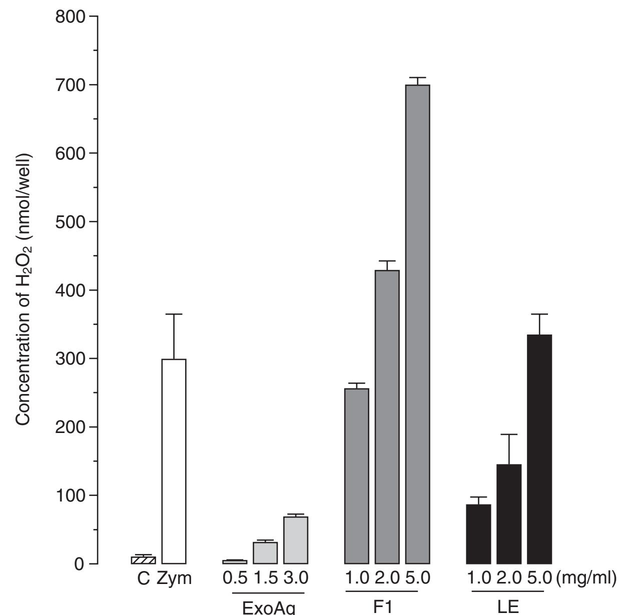

Figure 1 Reactive oxygen intermediates induced by cell derivatives of *Sporothrix schenckii*. Peritoneal macrophages (2×10^6 cells/ml) were cultured with *S. schenckii* derivatives at different concentrations (mg/ml). After 1 h of incubation of 37 °C in a mixture of 95% air and 5% CO_2, the reaction was interrupted with 10 μl of 4 N NaOH, and absorbance at 620 nm was measured using an automated microplate reader. Results are representative of four separate experiments and are presented as mean + standard error of the mean of triplicate observations. The results were statistically significant when compared with that of the control group (Zym) ($P < 0.05$). ExoAg, exoantigen; F1, alkali-insoluble fraction; LE, lipid extract.

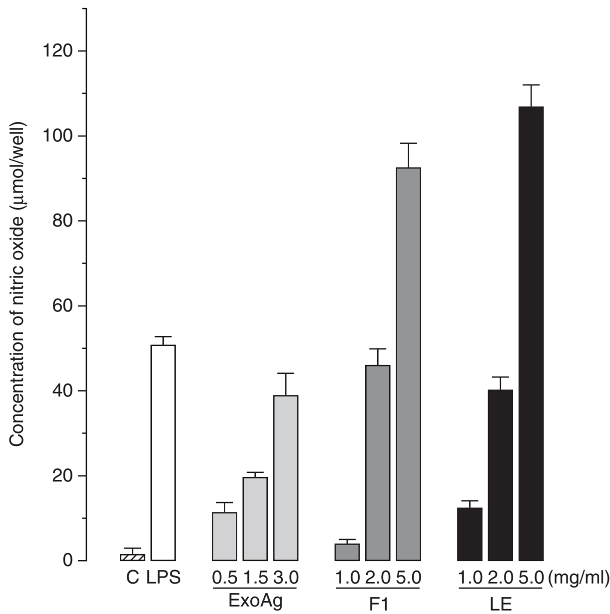

Figure 2 Reactive nitrogen intermediates induced by cell derivatives of *Sporothrix schenckii*. Peritoneal macrophages (5×10^6 cells/ml) were cultured with *S. schenckii* derivatives at different concentrations (mg/ml). The cells were incubated for 24 h before stimulation, and 50 µl aliquots of culture supernatant were mixed with 50 µl of Griess reagent and incubated at room temperature for 10 min, and the colour reaction was determined at 540 nm in an enzyme-linked immunosorbent assay reader. Supernatants from quadruplicate cultures were assayed in four experiments and reported as the mean NO_2^- concentration ± standard error of the mean. The results were statistically significant when compared with that of the control group (lipopolysaccharide (LPS)) ($P < 0.05$). ExoAg, exoantigen; F1, alkali-insoluble fraction; LE, lipid extract.

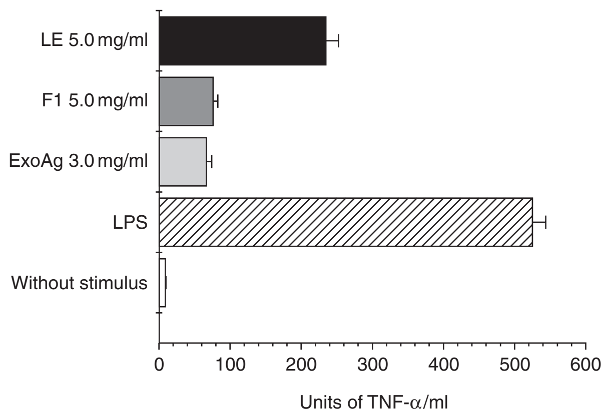

Figure 3 Units of tumour necrosis factor-α (TNF-α) in supernatants from peritoneal macrophage released by cell derivatives of *Sporothrix schenckii*. L929 cells were incubated with supernatants obtained from a macrophage culture. Cell survival was assessed by fixing and staining the cells with crystal violet, dissolving the stained cells in sodium dodecyl sulphate (SDS) and reading the absorbance of each well at 490 nm in an enzyme-linked immunosorbent assay reader. TNF-α units were calculated using a standard curve obtained with recombinant TNF-α constructed for each test run. To prove the presence of TNF-α in the culture supernatants, the preparations were previously incubated with rabbit anti-r TNF-α immune serum, and the test samples were added to L929. ExoAg, exoantigen; F1, alkali-insoluble fraction; LE, lipid extract.

induction of NO synthesis in IFN-γ and/or LPS-stimulated murine peritoneal macrophages and regulates NO synthesis *in vivo* [24, 25]. These results clearly showed this relation with respect to TNF-α and NO released when macrophages are in contact with LPS and LE from the fungus.

Treatment of macrophages with fungus derivatives

Macrophages are able to ingest particles and soluble substances. Ingestion of particles by macrophages is preceded by their attachment to the macrophage membrane. When previously treated with LE, macrophages completely inhibit the phagocytosis process (Figs 4 and 5). These results were not observed with F1 or ExoAg. Once again, these results demonstrate a differential role of LE, indicating that the binding involved a recognition mechanism for lipid compounds on the fungal cell wall. A positive correlation between NO concentration and *S. schenckii*-induced phagocytosis was clearly shown, as NO has been reported to be involved in the regulation of pseudopodium formation, phagocytosis and adhesion in macrophages through the reorganization of actin [26].

Discussion

The microbicidal activity of macrophages in general depends on their ability to both phagocytose organisms

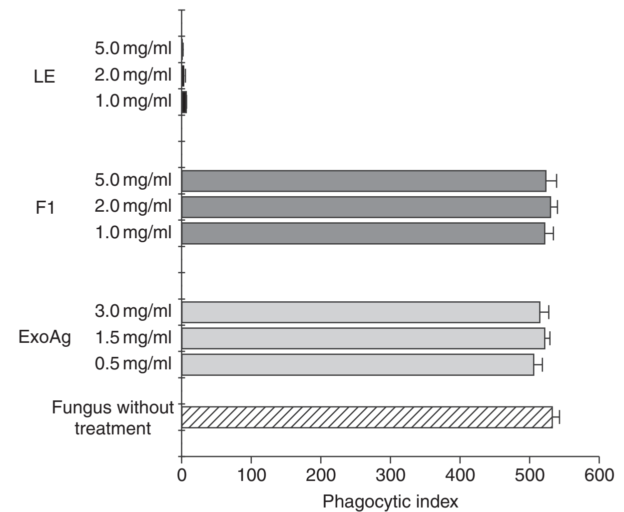

Figure 4 Effect of *Sporothrix schenckii* derivatives on phagocytosis inhibition. The derivatives were added at different concentrations to the slide-adhered macrophages from peritoneal exudate cells (PECs) (1.5×10^6 cell/ml). After washing, a yeast suspension of 2.5×10^7–5.0×10^7 cells per ml was added. Cells were stained with May–Grünwald–Giemsa. The percentage of macrophages phagocytosing at least one yeast cell (P) and the average number of yeasts in these macrophages (F). The phagocytic index (I) was determined by multiplying P and F [21]. ExoAg, exoantigen; F1, alkali-insoluble fraction; LE, lipid extract.

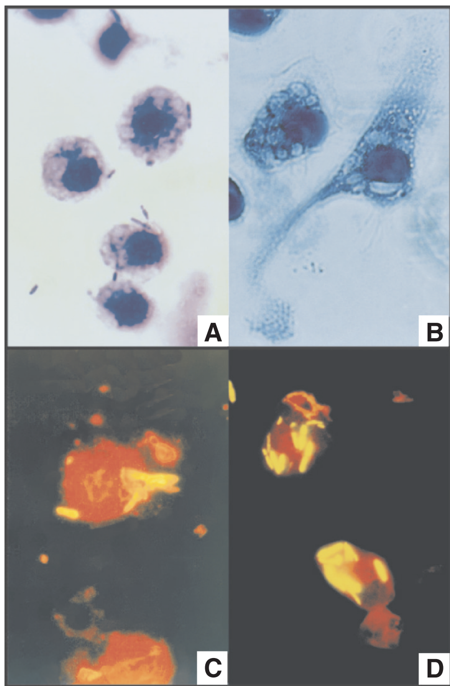

Figure 5 Photomicrograph illustrating the effect of *Sporothrix schenckii* derivatives on phagocytosis inhibition. The lipid extract (LE) at a concentration of 5.0 mg/ml was added to the slide-adhered macrophages from peritoneal exudate cells (PECs) (1.5×10^6 cell/ml). After washing, a yeast suspension of $2.5 \times 10^7 - 5.0 \times 10^7$ cells per ml was added. The derivative was subjected to previous LE treatment (A and C) or not (B and D) and stained with May–Grünwald–Giemsa (A and B) or fluorescein isothiocyanate (C and D).

and kill them once ingested. One mechanism by which macrophages kill and degrade ingested organisms is through the conversion of ambient oxygen to superoxide ion (O_2^-), H_2O_2, hydroxyl radical ($OH^\bullet$) and singlet oxygen ($^1O_2^\bullet$) [27, 28]. Under normal physiological conditions, H_2O_2 is generated in small quantities and is rapidly used or degraded, but long exposures and high concentrations of H_2O_2 can destroy biological structures and lead to irreversible cell damage [29]. In the present study, the F1 fraction was found to be a potential liberator of H_2O_2, while the LE preferentially induced NO. These results indicate that distinct fractions stimulated PECs, both probably contributing to the response occurring in sporotrichosis. Studies currently underway in our laboratory on a previous animal model (intravenous inoculation with 10^6 *S. schenckii* yeasts [30]) have shown that the F1 fraction induces a granulomatous reaction, whereas ExoAg participates in the humoral immune response. The importance of reactive oxygen metabolites in macrophage microbicidal activity is suggested by the evidence that peritoneal macrophages produce reactive oxygen metabolites upon activation or exposure to pathogens [31] and that macrophage activity is inhibited by free radical scavengers, such as superoxide dismutase, an enzymatic O_2^- scavenger and catalase and an enzymatic scavenger of H_2O_2 [32].

The reactive intermediates of nitrogen are currently considered to play an important role in the physiological and pathological process. Thus, increased NO production by macrophages may alter the balance between host defence mechanisms and pathogen virulence, thereby decreasing host susceptibility to the infectious process. Reactive oxygen and nitrogen species may play different roles in cells, depending on the relative concentrations of each and the cell sites accessible to these species. For example, H_2O_2 permeates cell membranes faster than $O_2^{\bullet-}$, and $NO^{\bullet-}$ permeates more rapidly than peroxynitrite. The chemical properties of the surrounding intra- or extracellular milieu are important because reactions of reactive oxygen and nitrogen species with oxygen, transition metals or each other can compete with reactions at cellular target sites [33]. NO is not a strong oxidant; however, NO reacts at a nearly diffusion-limited rate with superoxide to form a strong oxidant, peroxynitrite. Peroxynitrite is formed by activated inflammatory cells and agonist-stimulated endothelial cells and has been found to oxidize several biological molecules and to nitrate free or protein tyrosine residues and other phenolics [34, 35].

The precise role of macrophages in sporotrichosis is unknown. The monocytes/macrophages are important cells in host defence by their **your** direct microbicidal capacity or synthesis of regulatory proteins such as cytokines. In previous studies, we demonstrated the participation of the cellular immune response in systemic infection caused by *S. schenckii* [30, 36] and modifications in the release of cytokines such as IL-1 and TNF-α by adherent cells in this mycotic infection [20].

Macrophages activated by IFN-γ, TNF-α or LPS produce two kinds of reactive products characterized by their cytotoxic activity: reactive oxygen intermediates and reactive nitrogen intermediates [37]. In this report, the production of TNF-α was mainly detected with LE, similar to the results observed with the NO mediator. This was expected, because for the induction of *iNOS* gene expression, LPS-derived activation of a transcription factor, nuclear factor-kB (NF-kB) occurs, which translocates from the cytosol to the nucleus and binds to region 1 in the promoter of the *iNOS* gene [38], and many LPS-responsive genes, including those of TNF-α, are known to share recognition sequences that can be recognized by NF-kB for the activation of gene expression [39]. On this basis, the results of determination of NO and TNF-α using LE agree with the high liberation of NO and TNF-α in macrophage cultures.

We still know very little about the molecular mechanisms of innate immune recognition and the receptors involved. From the little that we do know, it is clear that innate immune recognition is mediated by structurally and functionally diverse receptors that can directly trigger a variety of host defence mechanisms such as complement activation, phagocytosis and expression of pro-inflammatory

genes. Over the last 3 years, there have been significant advances in our understanding of the biology of Toll-like receptors (TLR), mammalian homologues of Toll from *Drosophila*. This discovery of mammalian homologues of the *Drosophila* Toll receptor protein attracted interest in the role of these proteins in innate immunity with different ligand specificities [40]. Several features of TLR-4 have linked this protein to innate immune responses. The overexpression of a constitutively active TLR-4 protein drives NF-kB activation, B7.1 expression and cytokine production (IL-1, IL-6 and IL-8) in transfected THP-1 monocytes [41]. On the other hand, in studies in which the localization of TLR-2 was monitored after macrophage stimulation with zymosan, a presumed TLR-2 ligand, TLR-2 was shown to localize first to phagocytic cups and subsequently to phagosomes upon phagocytosis of zymosan. This striking observation suggests that macrophages use TLRs to determine the content of phagosomes [42]. *S. schenckii* LE plays an important role in the pathogenicity of this mycosis, inhibiting the phagocytic process. Macrophages treated with LE inhibit the phagocytosis process, an interesting result representing the first report about a compound of the fungus *S. schenckii* that presents this activity. The possible association between the results of the present study and this superfamily of TLRs could be tested in a future study, yielding information about inflammation and host defence, and probably opening new perspectives for the treatment of this mycosis.

In conclusion, the cell-wall lipid of this fungus could be considered as a virulence factor, and, on the other hand, macrophages produce cytotoxins linked to the activation of reactive species.

Acknowledgments

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