

Chloroquine is therapeutic in murine experimental model of paracoccidioidomycosis

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Received 5 December 2006; revised 5 February 2007; accepted 2 March 2007.
First published online April 2007.

DOI:10.1111/j.1574-695X.2007.00243.x

Editor: Alex van Belkum

Keywords

Paracoccidioides brasiliensis; chloroquine; iron; hydrogen peroxide; nitric oxide; cytokines.

Abstract

Chloroquine, due to its basic properties, has been shown to prevent the release of iron from holotransferrin, thereby interfering with normal iron metabolism in a variety of cell types. We have studied the effects of chloroquine on the evolution of experimental paracoccidioidomycosis by evaluating the viable fungal recovery from lung, liver and spleen from infected mice and H₂O₂, NO production, tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-10 levels and transferrin receptor (TfR) expression from uninfected and infected peritoneal macrophages. Chloroquine caused a significant decrease in the viable fungal recovery from all organs tested, during all periods of evaluation. Peritoneal macrophages from chloroquine-treated infected mice showed higher H₂O₂ production and TfR expression, and decreased levels of NO, endogenous and stimulated-TNF- α , IL-6 and IL-10 during the three evaluated periods. However, despite its suppressor effects on the macrophage function, the chloroquine therapeutic effect upon murine paracoccidioidomycosis was probably due to its effect on iron metabolism, blocking iron uptake by cells, and consequently restricting iron to fungus growth and survival.

Introduction

Paracoccidioidomycosis is a human systemic mycosis that is endemic in Latin America, and constitutes one of the most prevalent deep mycoses in endemic regions. This disease is caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus that develops as yeast at body temperature and as a mycelium at room temperature (Restrepo, 1985; Franco *et al.*, 1989). Ingested conidia or yeast-form of *P. brasiliensis* readily multiply inside murine alveolar or peritoneal macrophages; however, if they were activated by cytokines, such as interferon (IFN)- γ , the multiplication was limited and conidia or yeast cells were killed (Brummer *et al.*, 1989; Cano *et al.*, 1992). Similarly, the interaction of human phagocytic cells and yeast-form of *P. brasiliensis* was studied *in vitro* and yeast cells were readily ingested by monocytes or monocyte-derived macrophages. However, these cells allowed *P. brasiliensis* to multiply, unless they were activated with IFN- γ or culture supernatants from concanavalin-A-stimulated mononuclear cells, suggesting that this activation could be responsible for iron restriction

and consequent decrease in the survival of *P. brasiliensis* within cells (Moscardi-Bacchi *et al.*, 1994).

The mechanisms used by *P. brasiliensis* yeasts to survive and to multiply within monocytes or macrophages are poorly understood. Iron has been considered an essential element for microbial growth and has also been associated with virulence by promoting microorganism intracellular growth (Lewin, 1984). Chloroquine, a clinical drug, is best known as an antimalarial drug (Wellems, 1992; Slater, 1993), but it also affects several functions of macrophages (Ziegler & Unanue, 1982; Antoni *et al.*, 1986; Lang & Kaye, 1991; Zhu *et al.*, 1993). More recently, chloroquine has been used in the treatment of diseases associated with increased secretion of pro-inflammatory cytokines, such as rheumatoid arthritis and systemic lupus erythematosus (Fox, 1993; Wallace, 1994). It acts as a lysosomotropic agent by increasing lysosomal pH (Ziegler & Unanue, 1982) and, due to its basic properties, has been shown to prevent release of iron from holotransferrin (HTF) and ferritin, thereby interfering with normal iron metabolism, which is dependent on an

acidic environment (Forsbeck & Nilsson, 1983; Krogstad & Schlesinger, 1987; Sibille *et al.*, 1989; Byrd & Horwitz, 1991).

Moreover, studies showed that both mycelial and yeast forms of *P. brasiliensis* have a metabolic requirement for iron (Arango & Restrepo, 1988) and the intracellular conidium-to-yeast transformation of *P. brasiliensis* ingested by murine macrophage was inhibited when cells were treated with the iron chelator deferoxamine or activated with spleen-cell-culture supernatants from immunized mice. The HTF reversed this inhibitory effect of both deferoxamine and activated macrophages on *P. brasiliensis* intracellular transformation, indicating that iron restriction is one of the mechanisms by which activated macrophages control the intracellular transformation of ingested *P. brasiliensis* (Cano *et al.*, 1994). Likewise, studies in our laboratory have also demonstrated that deferoxamine or chloroquine inhibits the intracellular survival of *P. brasiliensis* yeasts within human monocytes by interfering with iron acquisition by the fungus (Dias-Melicio *et al.*, 2005, 2006). In addition, chloroquine treatment of Pb 18-infected mice significantly reduced the viable fungal recovery from lungs (Dias-Melicio *et al.*, 2006).

Based on these data, we undertook the current study to investigate chloroquine effects on the evolution of experimental paracoccidioidomycosis, as this drug prevents iron uptake by the cells and consequently makes iron unavailable to the intracellular fungus. For this, the viable fungal recovery from lung, liver and spleen from infected and chloroquine-treated infected mice was evaluated. Moreover, activity of peritoneal macrophages was evaluated by production of H_2O_2 and NO, levels of TNF- α , IL-6 and IL-10, and transferrin receptor (TfR-CD71) expression.

Materials and methods

Animals

Groups of six to eight BALB/c male mice (7–8 weeks old) were used for each period of infection. All the animals were purchased from the University of São Paulo (USP) and provided with water and sterilized food *ad libitum* throughout the experiment. All the procedures involving animals and their care were conducted in conformity with National and International Policies, and the Animals Ethics Committee of the Botucatu Medical School–UNESP approved the study.

Fungus

The highly virulent *P. brasiliensis* strain 18 (Pb 18) was used throughout this study. To ensure virulence, the isolate was used after three serial animal passages. Pb 18 yeast cells were then maintained by weekly subcultivation in the yeast-form cells at 35 °C on 2% glucose, 1% peptone, 0.5% yeast extract

and 2% agar medium (GPY medium) and used on the sixth day of culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS), pH 7.2. To obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10 s) (Peraçoli *et al.*, 1999). Yeast viability was determined by phase contrast microscopy; bright yeast cells were counted as viable and dark cells as not viable (Soares *et al.*, 2001). Fungal suspensions containing more than 95% viable cells were used in the experiments.

Paracoccidioides brasiliensis infection and chloroquine treatment

Mice were infected with 1×10^6 Pb 18 yeast cells in 100 μ L of sterile PBS by the caudal intravenous route. Groups of uninfected and infected mice were treated daily with chloroquine (Sigma-Aldrich Inc., St Louis, MO), at two different doses (40 or 80 mg kg⁻¹, prepared in sterile PBS and filtered) by intraperitoneal injection for 8 weeks. Uninfected and infected control groups received an intraperitoneal injection of sterile PBS. All the mice groups were evaluated at the 2nd, 4th and 8th weeks after infection. In these periods, the viable fungal recovery from lung, liver and spleen of infected and chloroquine-treated infected mice was evaluated. Moreover, H_2O_2 and NO production, TNF- α , IL-6 and IL-10 levels and TfR expression from peritoneal macrophages of all groups were assayed.

Viable fungal recovery

Groups of infected and treated infected mice were sacrificed chronologically in the 2nd, 4th and 8th weeks after infection, and the organs removed. Briefly, lungs, liver and spleen were put in sterile tubes, weighed separately, macerated and homogenized in 2.0 mL of sterile PBS. Aliquots of 100 μ L of each homogenate were plated in triplicate on brain–heart infusion (BHI) agar medium (Difco Laboratories) containing 4% normal horse serum and 5% *P. brasiliensis* strain 192 culture filtrate (v/v), the latter being the source of growth-promoting factor (Singer-Vermes *et al.*, 1992). Inoculated plates were incubated at 35 °C in sealed plastic bags to prevent drying. After 10 days the number of CFU in each plate was counted, and the results were expressed as CFU per gram of tissue.

Macrophage cultures

Peritoneal resident cells were obtained from uninfected, chloroquine-treated uninfected, infected and chloroquine-treated infected mice by washing their peritoneal cavities with 10 mL ice-cold RPMI 1640 (Sigma-Aldrich). The cells from each group of mice were centrifuged (180 g, 10 min, 4 °C), and washed twice with RPMI-1640 tissue culture

medium. After, the cells were prepared in complete tissue culture medium (CTCM) which consisted of RPMI 1640 tissue culture medium, supplemented with 2 mM L-glutamine (Sigma-Aldrich), 10% heat-inactivated foetal calf serum, 20 mM HEPES (Sigma-Aldrich) and 40 µg mL⁻¹ gentamicin (Gibco Laboratories, Grand Island, NY). The macrophages and total cells were counted with neutral red (0.02%) and trypan blue staining respectively, and the concentrations were adjusted according to the different assays. Suspensions of 100 µL per well, containing 2×10^5 macrophages each, were dispensed into 96-well flat-bottomed plates (Nunc, Life Tech Inc., MD) for H₂O₂ and NO assays. Suspensions of 1000 µL well⁻¹, containing 1×10^6 macrophages mL⁻¹, were dispensed into in 24-well flat-bottomed plates (Nunc) for culture supernatant cytokine measurement assays. After incubation for 2 h at 37 °C in 5% CO₂, nonadhering cells from both plates were removed by aspiration and each well was rinsed twice with CTCM. After adherence, the macrophages were cultured in CTCM at 37 °C in 5% CO₂ for 18 h, alone or stimulated with lipopolysaccharide (20 µg mL⁻¹). In all experiments the cell suspensions consisted of at least 98% macrophages.

Macrophage hydrogen peroxide (H₂O₂) release

The H₂O₂ production was measured by the modified HRP-dependent phenol red oxidation microassay according to Pick & Keisari (1980) and Pick & Mizel (1981). Briefly, after cells were cultured as described earlier, the supernatants were collected for the NO assay. To the macrophage monolayers were added a phenol red buffer with 100 µL of phenol red solution containing 5.5 mM dextrose, 0.56 mM phenol red (Sigma-Aldrich), and 0.01 mg mL⁻¹ horseradish type II (HRP type II–Sigma-Aldrich). In cocultures, 10 µL phorbol myristate acetate (PMA) (1 µg mL⁻¹; Sigma-Aldrich) was added. The plates were incubated in a humidified chamber for 1 h in 5% CO₂ at 37 °C. The reaction was stopped by addition of 10 µL of 1 N NaOH and the absorbance at 620 nm was determined by a micro-ELISA reader (MD 5000, Dynatech Laboratories Inc., Chantilly, VA). The conversion of absorbance to nanomolar concentrations of H₂O₂ was obtained from a standard curve. All determinations were performed in triplicate and expressed as nanomolar concentrations of H₂O₂.

Macrophage NO production

NO production was quantified by the accumulation of nitrite in the supernatants by the standard Griess reaction (Green, 1981). Briefly, 100 µL of the supernatants collected from 96-well macrophage cultures were added with an equal volume of Griess reagent containing 1% sulfanilamide (Sigma-Aldrich) diluted in 5% of H₃PO₄, and 0.1% N-(1-naphthyl)ethylenediamine (NEED–Sigma-Aldrich). The

absorbance at 540 nm was determined by a micro-ELISA reader (MD 5000, Dynatech Laboratories). Conversion of absorbance to micromolar concentrations of NO was obtained from a standard curve using a known concentration of NaNO₂ diluted in distilled H₂O. All determinations were performed in triplicate and expressed as micromolar concentrations of NO.

Measurement of cytokines

Peritoneal macrophage culture supernatants were separated from cell debris by centrifugation at 1000 g for 15 min, and stored at –70 °C. Next, the levels of TNF-α, IL-6 and IL-10 were measured by capture ELISA using Kit DuoSet (R&D Systems, Minneapolis, MN). The ELISA procedure was performed according to the manufacturer's protocol. The concentrations of cytokines were determined with reference to a standard curve for serial twofold dilutions of murine recombinant cytokines. The absorbance values were measured at 492 nm using a micro-ELISA reader (MD 5000, Dynatech Laboratories). The lower limit of each recombinant standard curve detection was 15.62, 7.8 and 15.62 pg mL⁻¹ for TNF-α, IL-6 and IL-10, respectively.

TfR expression

The peritoneal total cell suspensions containing 1×10^6 total cells mL⁻¹ were labeled with rat antimouse CD71-FITC (Caltag Laboratories, Burlingame, CA) and incubated for 1 h on ice in the dark. Nonspecific signals were calculated and attenuated by incubation with the rat IgG2a isotype control-FITC (Caltag Laboratories) as a secondary antibody in the absence of the primary antibody. After, the cells were washed in PBS, 100 µL of fixative solution consisting of 5% formaldehyde in buffer (Becton Dickinson, San Jose, CA) was added, and the solution analyzed by flow cytometry on a FACSCalibur Cytometer (Becton Dickinson) using WIN MIDI and CELL QUEST software.

Statistical analysis

Results were statistically analyzed by ANOVA using GRAPHPAD software (GraphPad InStat 3.05, San Diego, CA) and compared using the Tukey–Kramer test (Zar, 1984; Godfrey, 1985) with the level of significance set at $P < 0.05$.

Results

Chloroquine strongly decreases the number of viable fungi from lung, liver and spleen of Pb 18-infected mice

The treatment of mice with chloroquine reduced the numbers of *P. brasiliensis* in the lungs, liver and spleen during all evaluated periods (Fig. 1). At the first period of evaluation

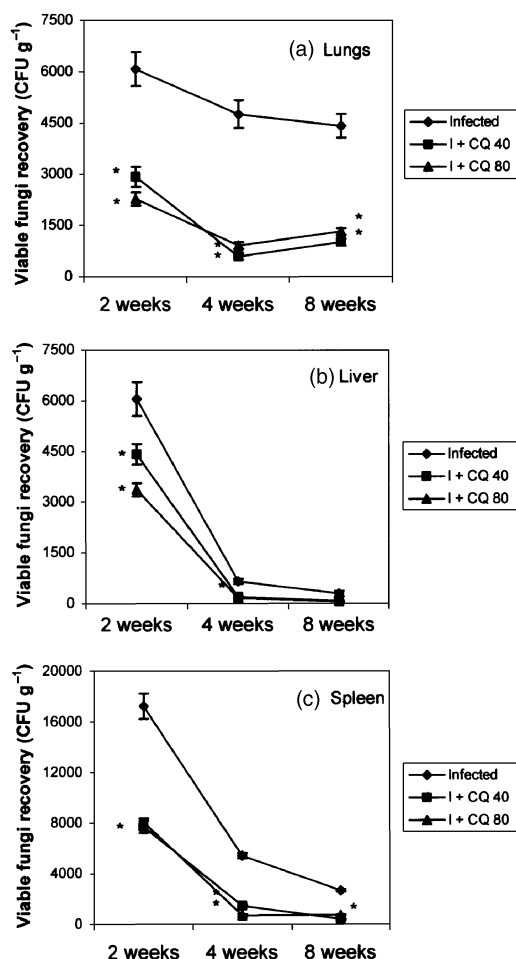


Fig. 1. Chloroquine strongly decreases the number of viable fungi from Pb 18-infected mice. The mice were inoculated intravenously with 10^6 yeasts cells of Pb 18, and groups were treated daily with two doses of chloroquine (40 and 80 mg kg^{-1}) over three different periods (2, 4 and 8 weeks after infection). The recovery of CFU g^{-1} from lungs (a), liver (b) and spleen (c) from all groups is shown. The results are the means of six to eight animals and range bars represent SEs of the means and are representative of two experiments. Statistical significance between groups is indicated for each period.

(2nd week), the infected group showed a high recovery of CFU from lung and the chloroquine-treated groups showed a great reduction in the viable fungal recovery at two doses used (40 and 80 mg kg^{-1}), both statistically different from infected group ($P < 0.01$) (Fig. 1a). Although infected mice showed a slight decrease in viable fungal recovery from lungs during the 4th and 8th weeks postinfection, the chloroquine effect on treated groups with two doses was more pronounced during these two periods (4th and 8th weeks) ($P < 0.01$).

The levels of fungal recovery from liver were similar to those obtained from lungs at the 2nd week in infected groups (Fig. 1b). In this period, the chloroquine-treated

infected mice showed a significant reduction in the CFU recovery, with doses of 80 mg kg^{-1} ($P < 0.01$) and 40 mg kg^{-1} ($P < 0.05$). At the 4th week, there was still a significant decrease in the number of viable fungal recovery from liver in the chloroquine-treated mice ($P < 0.05$), although the infected group did show some reduction in fungal load. The results from about the 8th week showed a reduction of CFU in all groups, including the infected ones that did not receive chloroquine treatment. In this period (8th week), there was no difference between groups, indicating a tendency to resolution of infection in this organ.

In the spleen at the 2nd week, the levels of CFU detected in the infected group were two times higher than in the liver and lung (Fig. 1c). However, the chloroquine treatment at two doses significantly diminished this recovery ($P < 0.01$). By the 4th week, chloroquine-treated groups still showed a significant reduction in CFU recovery ($P < 0.05$), although the infected group had presented reduction in fungal load, too. The results around the 8th week showed a reduction of CFU in all groups, including the infected ones that did not receive chloroquine treatment, indicating a tendency to resolution of infection in this organ, too, although there was still a significant difference between infected and chloroquine-treated (40 and 80 mg kg^{-1}) infected mice.

In relation to viable fungal recovery from infected treated mice, there were no differences in the groups treated with 40 – 80 mg kg^{-1} of chloroquine at any point of treatment, in any organ evaluated.

Chloroquine increases H_2O_2 production by peritoneal macrophages

At the 2nd week, macrophages from infected mice showed higher H_2O_2 levels compared with the uninfected mice ($P < 0.01$) (Fig. 2). Macrophages from chloroquine-treated infected mice (40 and 80 mg kg^{-1}) increased H_2O_2 production compared with the infected mice ($P < 0.01$). Activation by lipopolysaccharide ($20 \mu\text{g mL}^{-1}$; data not shown) and PMA ($1 \mu\text{g mL}^{-1}$) increased the H_2O_2 production from uninfected and infected macrophages ($P < 0.01$).

At the 4th week (Fig. 2), macrophages from infected mice maintained high H_2O_2 levels compared with the first period, and macrophages from chloroquine-treated infected mice increased the H_2O_2 production in relation to the infected ones and in relation to the first period (chloroquine 80 mg kg^{-1}).

During the 8th week, the results were similar to those shown at the 4th week, with chloroquine-treated infected groups producing higher H_2O_2 levels than infected ones, and a slight decrease in all groups (data not shown).

The uninfected mice treated with two doses of chloroquine, during the three periods of evaluation, showed increases in H_2O_2 levels ($P < 0.01$). Uninfected mice and

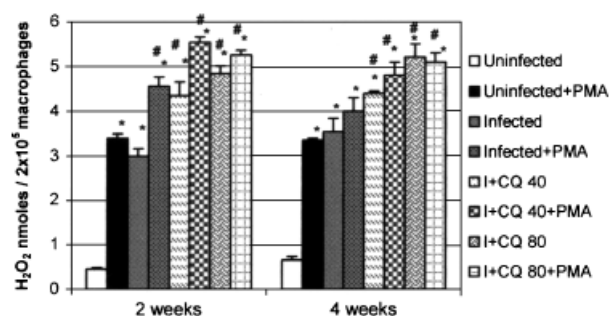


Fig. 2. Chloroquine increases H_2O_2 production by peritoneal macrophages. H_2O_2 production by peritoneal macrophages from uninfected, infected and chloroquine-treated infected mice (chloroquine 40 and chloroquine 80) at 2 and 4 weeks after infection, stimulated or not with PMA ($1 \mu\text{g mL}^{-1}$) is shown. Each bar represents the mean of a pool from six to eight animals tested in triplicate and range bars represent SEs of the means. The results are representative of two experiments. Statistical significance between groups is indicated for each period. *vs. uninfected and # vs. infected group.

chloroquine-treated uninfected mice (40 and 80 mg kg^{-1}) showed a mean \pm SEM production of H_2O_2 of 0.45 ± 0.03 , $1.35 \pm 0.04^*$, and $3.69 \pm 0.4^*$ nmol at the 2nd week, respectively; 0.66 ± 0.08 , $2.97 \pm 0.04^*$ and $3.21 \pm 0.2^*$ nmol at the 4th week, respectively; and 0.53 ± 0.09 , $3.9 \pm 0.2^*$ and $4.2 \pm 0.7^*$ nmol at the 8th week, respectively (asterisks indicate a significant difference). Activation by lipopolysaccharide ($20 \mu\text{g mL}^{-1}$) and PMA ($1 \mu\text{g mL}^{-1}$) increased the H_2O_2 production from all these groups.

Chloroquine diminishes NO production by peritoneal macrophages

Macrophages from infected mice, at the 2nd week, increased NO levels compared with the uninfected mice, as shown in Fig. 3 ($P < 0.01$). However, macrophages from chloroquine-treated infected mice (40 and 80 mg kg^{-1}) decreased the NO levels compared with the infected ones ($P < 0.01$). Activation by lipopolysaccharide ($20 \mu\text{g mL}^{-1}$) increased NO production in all groups ($P < 0.01$).

At the 4th week (Fig. 3), macrophages from infected mice and from chloroquine-treated infected mice had decreased NO levels compared with the first period ($P < 0.05$), and the difference between infected mice and from chloroquine-treated infected mice continued to be detected ($P < 0.01$). The cells from all groups had decreased NO production when activated by lipopolysaccharide ($20 \mu\text{g mL}^{-1}$) in relation to the first period ($P < 0.05$).

During the 8th week, the results were similar to those in the 4th week, with infected and chloroquine-treated infected groups producing lower NO levels, and with a decrease in NO production when activated with lipopolysaccharide ($20 \mu\text{g mL}^{-1}$) (data not shown).

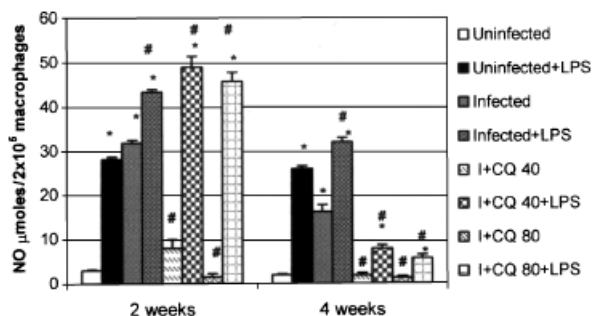


Fig. 3. Chloroquine diminishes NO production by peritoneal macrophages. NO production by peritoneal macrophages from uninfected, infected and treated infected mice (chloroquine 40 and chloroquine 80) at 2 and 4 weeks after infection, stimulated or not with lipopolysaccharide ($20 \mu\text{g mL}^{-1}$) is shown. Each bar represents the mean of a pool from six to eight animals tested in triplicate and range bars represent SEs of the means. The results are representative of two experiments. Statistical significance between groups is indicated for each period. *vs. uninfected and # vs. infected group.

The uninfected mice treated with two doses of chloroquine showed a slight increase in NO levels compared with uninfected mice in the 2nd and 4th weeks, and similar levels in the 8th week. Uninfected mice and chloroquine-treated uninfected mice (40 and 80 mg kg^{-1}) showed a mean \pm SEM increase in NO of 2.7 ± 0.02 , 3.4 ± 0.2 and $4.7 \pm 0.03^*$ μmol at the 4th week, respectively; and 2.4 ± 0.09 , 2.6 ± 0.2 and $2.7 \pm 0.02 \mu\text{mol}$ at the 8th week, respectively (asterisks indicate a significant difference). The chloroquine-treated uninfected mice (40 and 80 mg kg^{-1}) produced lower NO levels than uninfected mice when activated with lipopolysaccharide ($20 \mu\text{g mL}^{-1}$).

TfR expression

Figure 4 shows the results of macrophage TfR expression. Uninfected mice macrophages presented a higher CD71 expression percentage than did the infected ones ($P < 0.001$) during the three different periods. The chloroquine-treated infected mice showed an increase in macrophage TfR expression at the 2nd and 4th weeks compared with infected ones ($P < 0.05$). Data from the 8th week were similar to those presented in the 4th week (data not shown). However, the macrophages from chloroquine-treated uninfected mice presented a decreased TfR expression compared with uninfected mice, indicating that this modulation of CD71 expression is not directly due to the chloroquine effect. This modulation could be due to NO production that was negatively correlated with TfR expression (Fig. 5).

Chloroquine diminishes $\text{TNF-}\alpha$ and IL-6 levels

Figure 6 shows the effect of chloroquine treatment on endogenous and lipopolysaccharide-stimulated $\text{TNF-}\alpha$ (Fig. 6a), IL-6 (Fig. 6b) and IL-10 (Fig. 6c) levels,

respectively, obtained from macrophage cultures. At the 2nd week, the infected mice macrophages did not produce endogenous TNF- α and these cells did not respond to lipopolysaccharide activation (Fig. 6a) ($P < 0.001$). At the 4th and 8th weeks, infected mice macrophages presented and increase in TNF- α and responded to lipopolysaccharide ($P < 0.001$). Chloroquine treatment significantly decreased the endogenous and lipopolysaccharide-stimulated TNF- α .

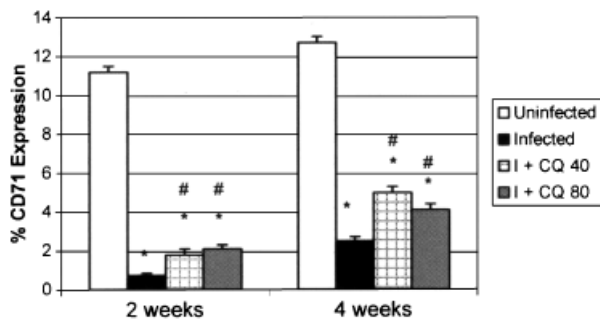


Fig. 4. Transferrin receptor expression (CD71) percentage in peritoneal macrophages from uninfected, infected and treated infected mice (chloroquine 40 and chloroquine 80) at 2 and 4 weeks after infection is shown. Each bar represents the mean of a pool from six to eight animals tested in triplicate and range bars represent SEs of the means. The results are representative of two experiments. Statistical significance between groups is indicated for each period. *vs. uninfected and # vs. infected group.

levels of infected mice macrophages, mainly at the 4th week ($P < 0.001$) (Fig. 6a) and similar results were observed in the 8th week of evaluation (data not shown).

The uninfected mice treated with two doses of chloroquine showed decrease in TNF- α levels compared with uninfected mice during the three evaluation periods ($P < 0.05$). Uninfected mice and chloroquine-treated uninfected mice (40 and 80 mg kg⁻¹) showed a decrease in TNF- α (mean \pm SEM) of 42 ± 1.7 , 39 ± 2 and $29.7 \pm 0.9^*$ pg mL⁻¹ at the 2nd week, respectively; 43.4 ± 1.9 , $32.5 \pm 0.5^*$ and $25.3 \pm 0.9^*$ pg mL⁻¹ at the 4th week, respectively; and 42.7 ± 2 , $23.8 \pm 2.1^*$ and $20.2 \pm 1.7^*$ pg mL⁻¹ at the 8th week, respectively. The chloroquine-treated uninfected mice (40 and 80 mg kg⁻¹) produced lower TNF- α than uninfected mice when activated with lipopolysaccharide (20 μ g mL⁻¹).

In relation to IL-6, chloroquine significantly diminished the endogenous levels of this cytokine from infected mouse macrophages during the three periods and lipopolysaccharide-stimulated IL-6 levels in the second and third periods (Fig. 6b). The uninfected mice treated with two doses of chloroquine showed decrease in IL-6 levels compared with uninfected mice during the three evaluation periods.

The endogenous IL-10 levels were similarly low in uninfected and infected groups, and chloroquine treatment decreased infected macrophage IL-10 levels compared with only infected groups during the three periods (Fig. 6c). The uninfected mice treated with two doses of chloroquine

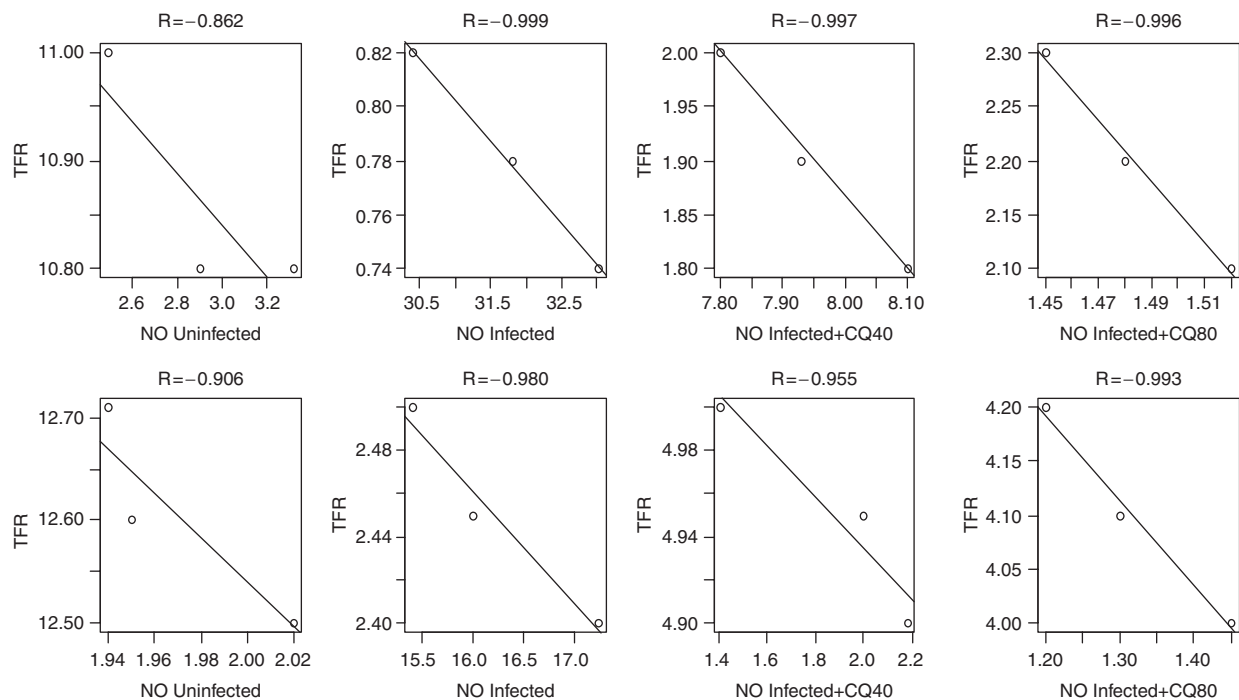


Fig. 5. Negative correlation between NO levels (μ mol per 2×10^5 cells) and Tfr expression (CD71%) in peritoneal macrophages from uninfected, infected and treated infected mice at 2nd (top) and 4th (bottom) weeks after infection are shown.

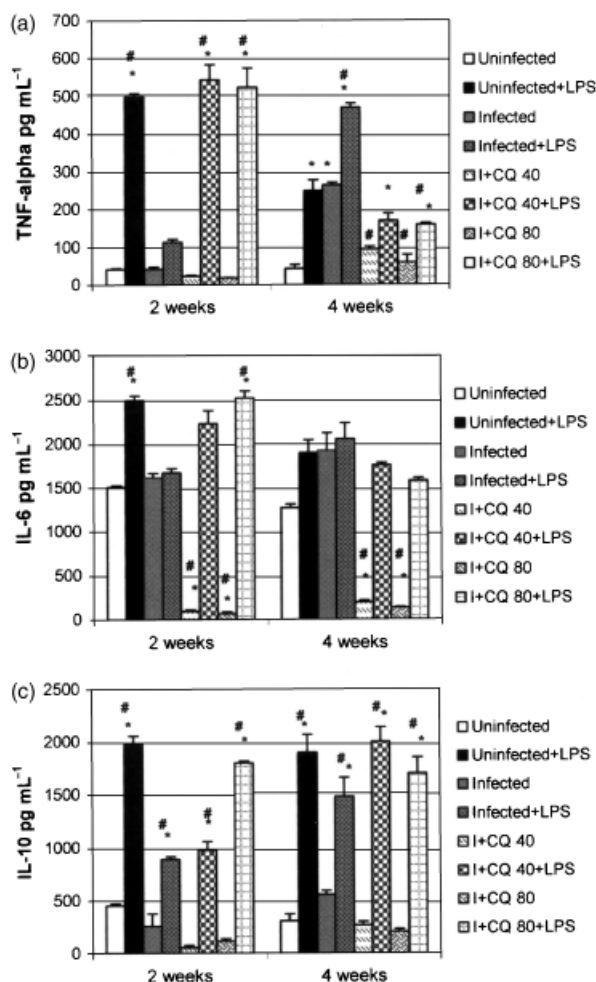


Fig. 6. Chloroquine diminishes TNF- α , IL-6 and IL-10. TNF- α (a), IL-6 (b) and IL-10 (c) peritoneal macrophage culture levels from uninfected, infected and treated infected mice (chloroquine 40 and chloroquine 80) at 2 and 4 weeks after infection, stimulated or not with lipopolysaccharide (20 μ g mL⁻¹) are shown. Each bar represents the mean of a pool from six to eight animals tested in triplicate and range bars represent SEs of the means. The results are representative of two experiments. Statistical significance between groups is indicated for each period. *vs. uninfected group and # vs. infected group.

showed decrease in IL-10 levels compared with uninfected mice only during the last evaluation period.

Discussion

This study demonstrated for the first time that chloroquine was effective in decreasing the fungal load of lung, liver and spleen from infected *P. brasiliensis* mice. We believe that this effect was due to chloroquine action upon iron metabolism, as studies have shown that chloroquine diminishes the iron content in macrophages, liver and spleen of normal mice and iron-treated mice (Legssyer *et al.*, 1999, 2003). We have previously demonstrated the role of iron in *P. brasiliensis*

growth, showing that when monocytes were pretreated with deferoxamine, an iron chelator drug, *P. brasiliensis* survival was inhibited within cells and HTF was capable of reversing deferoxamine inhibitory effect by supplying iron to intracellular *P. brasiliensis* (Dias-Melicio *et al.*, 2005). We also showed that chloroquine inhibits the intracellular survival of *P. brasiliensis* in human monocytes by interfering with iron acquisition by the fungus, reinforcing the hypothesis that availability of intracellular iron is clearly required for the survival of this pathogen. This inhibitory chloroquine effect on *P. brasiliensis* survival was reversed by FeNTA, an iron compound that is soluble in the neutral to alkaline pH range, but not by HTF, which requires an acidic pH to release ferric ions (Dias-Melicio *et al.*, 2006).

By increasing lysosomal and endocytic vesicle pH, chloroquine interferes with intracellular iron metabolism in at least two major ways. First, this drug may inhibit the pH-dependent release of iron from HTF endocytized via transferrin receptors (TfR). Normally, TfR binds HTF on the cell surface, forming a complex that it is endocytized, and the endocytic vesicle is acidified. When the pH decreases to 5, all ferric ions dissociate from HTF and are transported across the vesicle membrane into the cytoplasm (Princiotta & Zapolski, 1975; Lestas, 1976; Byrd & Horwitz, 1989, 1991; Nunez *et al.*, 1990; Lane *et al.*, 1991; Newman *et al.*, 1994). Thus, HTF becomes ATF, an unsaturated transferrin, that remains bound to the TfR and is recycled to the cell surface and dissociates from the TfR (Dautry-Varsat *et al.*, 1983; Brummer *et al.*, 1989; Byrd & Horwitz, 1989, 1991; Alford *et al.*, 1991; Lane *et al.*, 1991; Newman *et al.*, 1994; Conrad *et al.*, 1999). Upon translocation across the endocytic vesicle membrane, iron enters the cytosolic labile iron pool and reaches equilibrium with iron storage (Jacobs, 1977; Roberts & Bomford, 1988; Lane *et al.*, 1991). Thus, chloroquine has been found to reduce markedly the release of iron from endocytized HTF in a variety of cell types, including mononuclear phagocytes and macrophages, without affecting receptor recycling (Octave *et al.*, 1979; Forsbeck & Nilsson, 1983; Swaiman & Machen, 1986; Baynes *et al.*, 1987).

The second way that chloroquine may interfere with intracellular iron metabolism is by blocking recycling of iron from ferritin. Studies have suggested that iron bound to ferritin is released to the intracellular intermediate labile iron pool after ferritin is degraded in lysosomes. By raising intralysosomal pH, chloroquine may block degradation of ferritin by acid proteases, by virtue of its ability to raise endocytic and lysosomal pH (Sibille *et al.*, 1989), blocking iron release from ferritin.

Thus, by interfering with the release of iron from these two major sources to the intermediate labile iron pool of phagocytes, chloroquine may decrease the availability of iron to intracellular *P. brasiliensis* and inhibit survival and

multiplication of this intracellular pathogen; subsequently it is killed, decreasing the fungal load.

Several studies have shown the modulation of intracellular iron pool by chloroquine and its inhibitory action on *in vitro* intracellular growth of *Mycobacterium avium*, *Cryptococcus neoformans*, *Penicillium marneffei*, *Leishmania* ssp., *Histoplasma capsulatum*, *Mycobacterium tuberculosis* and *P. brasiliensis* within murine and human phagocytes (Crowle & May, 1990; Newman *et al.*, 1994; Levitz *et al.*, 1997; Boelaert *et al.*, 2001; Dias-Melicio *et al.*, 2006). *In vivo*, chloroquine was tested in histoplasmosis and cryptococcosis murine models (Newman *et al.*, 1994; Levitz *et al.*, 1997), and the results were similar to those found in the present study, with a decrease in tissue fungal load also being obtained.

In our model, an important chloroquine effect detected was the increased H₂O₂ production by chloroquine-treated macrophages. These results could be explained by the effect of chloroquine on the inhibition of catalase activity (Legssyer *et al.*, 1999). This process could be involved in the decrease of fungal load, as some studies have suggested that this metabolite is important for the killing of *P. brasiliensis* (Cano *et al.*, 1995; Moreira *et al.*, 2001; Calvi *et al.*, 2003; Carmo *et al.*, 2006).

Another important chloroquine effect was upon inhibition of NO production. These results are in agreement with studies that demonstrated the inhibitory effect of chloroquine on inducible nitric oxide synthase (iNOS), which is responsible for NO production (Hrabák *et al.*, 1998; Park *et al.*, 1999; Musial & Eissa, 2001; Utaisincharoen *et al.*, 2002). An experimental paracoccidioidomycosis study has shown that this metabolite has a dual role in the experimental model; and it is essential for resistance, but overproduction is associated with susceptibility (Nascimento *et al.*, 2002). Bocca *et al.* (1998) also showed that the activation of NO production in *P. brasiliensis* infection contributes to the occurrence of the immunosuppression observed during the course of the infection, although some studies demonstrated that NO is important for the killing of the fungi (Gonzalez *et al.*, 2000). Thus, this inhibitory chloroquine effect on NO production, observed in our model, could have eliminated one of the major suppressor factors that influence the mice response, promoting the better response to infection.

An important role of NO in iron metabolism was described through its modulatory effect on the iron-regulatory proteins (IRP-1 and IRP-2). These are cytoplasmic proteins responsible for controlling cellular iron storage and uptake by interaction with specific nucleotide sequences, called iron-responsive elements (IREs), which are located in the 3'-untranslated region (UTR) of TfR mRNA as well as the 5'-UTRs of mRNA for ferritin. When iron in the intracellular transit pool is scarce, IRPs bind to IREs in

the 5'-UTRs of the ferritin mRNA and 3'-UTR of the TfR mRNA. Such binding inhibits translation of ferritin mRNA and stabilizes the mRNA for TfR, whereas the opposite scenario develops when iron in the transit pool is plentiful (Hentze & Kuhn, 1996; Richardson & Ponka, 1997). Studies showed that NO causes a significant decrease in IRP-2 binding to the IREs followed by IRP-2 degradation that resulted in a dramatic decrease in TfR mRNA levels (Kim & Ponka, 1999, 2002). Thus, this NO modulatory action could explain our results, in which TfR expression was negatively correlated with NO levels. Studies have shown that IFN- γ reduces the TfR expression on macrophages, decreasing cellular iron uptake, and consequently the iron availability (Byrd & Horwitz, 1989, 1993; Lane *et al.*, 1991). This process is mediated by an increase in NO levels from IFN-activated cells, and the NO effect on IRPs promotes the decrease in TfR expression (Kim & Ponka, 2000). However, we demonstrated that chloroquine-treated infected mice macrophages showed a decrease in NO levels that promoted an increase in TfR expression, which could induce an increase in intracellular iron. Even so, this process might be inhibited by the effect of chloroquine on the pH-dependent release of iron from HTF endocytized via transferrin receptors (TfR), with consequent decrease in iron intracellular content.

This study also investigated whether the chloroquine treatment was associated with changes in the levels of TNF- α , IL-6 and IL-10 produced by macrophages. Some studies have shown the inhibitory effect of chloroquine on cytokine production (Zhu *et al.*, 1993; Hong *et al.*, 2004; Rayne *et al.*, 2004). Our data demonstrated that chloroquine treatment significantly decreased IL-6 production. This process could be important given that monocytes from paracoccidioidomycosis patients produce high IL-6 levels during the active disease, and this cytokine is capable of deactivating macrophages with consequent reduction in microbicidal activity against microorganisms (Blanchard *et al.*, 1991; Bermudez *et al.*, 1992). Furthermore, it has been shown that IL-6 increases Pb 18 growth in human monocytes compared with untreated monocytes (Siqueira *et al.*, 2007), suggesting that this cytokine may contribute to the pathogenesis of infection with *P. brasiliensis* by promoting fungal growth during interaction with phagocytes.

Another cytokine that has a central role in paracoccidioidomycosis infection is TNF- α . Our results showed an eminent inhibitory chloroquine effect on TNF- α production, confirming previous studies showing that chloroquine is capable of diminishing macrophage TNF- α production (Hong *et al.*, 2004; Rayne *et al.*, 2004) by inhibiting TNF- α mRNA transcription (Zhu *et al.*, 1993). Although this cytokine is associated with granulomatous reaction formation (Soler & Bernaudin, 1993), in paracoccidioidomycosis several authors report high levels of TNF- α in serum (Silva *et al.*, 1995; Silva & Figueiredo, 1995; Fornari *et al.*, 2001)

and in peripheral blood mononuclear cell (PBMC) culture supernatants during active disease (Karhawi *et al.*, 2000). Thus, the capacity to produce high TNF- α levels might be involved in the pathogenesis of the disease rather than in the resistance, even though several works have shown that TNF- α is very important for human monocyte activation and for effective killing of *P. brasiliensis* (Calvi *et al.*, 2003). In experimental paracoccidioidomycosis models, persistent TNF- α production has been associated with resistance to *P. brasiliensis* infection, which is also essential for the control of fungal dissemination (Cano *et al.*, 1995; Bocca *et al.*, 1998; Souto *et al.*, 2000; Nascimento *et al.*, 2002). Considering these processes, in the present paper, the inhibitory chloroquine effect on TNF- α production could be one of the mechanisms by which chloroquine controls the infection promoting better control of the deleterious TNF- α effects on tissue, and the TNF- α participation in the resistance could be supplied by the effect of chloroquine on the iron metabolism interfering with iron acquisition by the fungus.

Our findings support the idea that the effect of chloroquine on murine experimental paracoccidioidomycosis is due to its capacity to interfere with intracellular iron availability that disabled its uptake by the fungus with consequent fungal death, as well as by its suppressor effects on the immune response. Up to now, no data have been available on the possible antifungicidal effect of chloroquine in the clinical situation. Thus, chloroquine could be utilized as a potential drug that could be administered in association with conventional paracoccidioidomycosis treatment.

Acknowledgements

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), process no. 02/03680-8, Brazil.

We thank Professor Cesar Augusto Taconeli (Department of Biostatistics/IB-UNESP) for the correlation graphs, Luiz Henrique Alquati for helpful technical assistance, and Valéria Alves da Silva for helpful assistance in flow cytometry assays.

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