Multinucleated giant cells (MGC) are a common feature of granulomatous lesions induced by intracellular pathogens such as fungi and mycobacteria. MGC are also formed in vitro from peripheral blood mononuclear cells (PBMC) by stimulation with cytokines and lectins (Okamoto et al. 2004, Nascimento et al. 2008). Considerable evidence suggests that MGC are formed by the fusion of cells in the mononuclear phagocyte lineage, particularly monocytes from human blood (Enelow et al. 1992a, Anderson 2000). The phenomenon of adherent macrophage fusion in both human and experimental animals is enhanced in vitro by the addition of cytokines (McInnes & Rennick 1988, Chensue et al. 1992), specific antibodies (Orentas et al. 1992), mitogens (Hassan et al. 1989) and conditioned medium (Mizuno et al. 2001, Nascimento et al. 2008). Interferon-gamma (IFN-γ) is essential for MGC generation by promoting cell clustering and cell-to-cell adhesion (Most et al. 1990, Fais et al. 1994). Antibodies against IFN-γ inhibit MGC formation both in vitro (Fais et al. 1994) and in vivo (Belosevic et al. 1989).

Helming and Gordon (2007) proposed that MGC formation in a granuloma could be induced by the cytokines and signals derived from bacteria, parasites or other foreign materials and that this local environment induces expression of fusogenic factors; therefore, the induction or modulation of MGC formation may be enhanced or suppressed depending on the cytokines present during the initial stimulus. In addition to IFN-γ other pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and granulocyte macrophage col-
Paracoccidioidiomycosis is caused by the dimorphic fungus *Paracoccidioides brasiliensis* and is the most prevalent systemic human mycosis in Latin American countries (Franco et al. 1993, Wanke & Londero 1994). The clinical manifestations of paracoccidioidomycosis are a chronic epithelioid granulomatous disease (Montenegro & Franco 1994) and an inflammatory response induced by the fungus that is supposed to prevent the fungus dissemination (Iabuki & Montenegro 1979, Franco et al. 1993). In some histopathology studies of paracoccidioidomycosis in patients and experimentally infected animals, granulomatous lesions with MGC containing fungal cells were present, which suggests that MGC have a role in the fungus-killing process (Parise-Forte et al. 2006). The specific role of MGC in inflammatory infection is not known; however, Enelow et al. (1992b) suggest that monocyte cultures stimulated with interleukin (IL)-3 and IFN-γ generate MGC with enhanced candidicidal activity.

An in vitro model may be critical for understanding the mechanisms of lesion formation in paracoccidioidomycosis. The in vitro generation of MGC using *P. brasiliensis* antigen (PbAg) with various pro and anti-inflammatory cytokines and the ability to assess the functional activity of these cells against *P. brasiliensis* strain 18 (Pb18) has not been previously investigated.

Thus, in this study, we evaluated the effects of GM-CSF in association with IFN-γ, IL-10, transforming growth factor beta (TGF-β) or TNF-α to modulate MGC generation from PbAg-stimulated human blood monocytes. The functional activity of the generated MCG was determined by in vitro challenge with Pb18.

**SUBJECTS, MATERIALS AND METHODS**

Healthy individuals - Twenty healthy blood donors between 20-50 years of age (mean age 31.5 ± 10.2 years) were recruited from the Botucatu Medical School at São Paulo State University, Brazil. The Botucatu Medical School Ethics Committee on human research approved the study and informed consent was obtained from all the blood donors.

Fungal strain - Pb18 was maintained in the yeast form at 35°C in glucose-peptone-yeast (GPY) culture medium (2% glucose, 1% peptone, 0.5% yeast extract and 2% agar) for six days. Yeast viability was determined by phase-contrast microscopy (Soares et al. 2001) and fungal suspensions containing more than 90% viable cells were used for the experiments.

Preparation of antigen - PbAg was prepared from Pb18 cultured on GPY agar at 35°C for five days as described previously (Nascimento et al. 2008). The fungal growth from three randomly selected tubes (about 300 mg, wet weight) was collected by gently scraping the surface. The cell mass was suspended in 1 mL of 0.15 M phosphate-buffered saline (PBS), pH 7.4, mixed for 30 s in a vortex mixer (Technal, São Paulo, Brazil) and immediately centrifuged at 5,000 rpm in an Eppendorf tabletop centrifuge (Sorvall MC 12 V; Dupont, Newtown, CT, USA) for 10 min. The resulting supernatant containing the cell-free antigen was filtered through a 0.22 µm membrane (Millipore Corporation, Bedford, MA). Protein concentration was determined by the Lowry et al. (1951) method.

Water extraction of yeast phase cells - The water extract of Pb18 was prepared according to Kurita et al. (1993) with modifications. Yeast phase cells of Pb18 strain were inoculated into a liquid GPY culture medium and incubated at 35°C for five days in a horizontal shaker at 140 rpm (Forma Scientific). Yeast cell growth was harvested, centrifuged at 2,500 rpm for 15 min and suspended in sterile distilled water at a density of 10% (v:v). The suspensions were heated at 120°C for 15 min and allowed to stand at room temperature (RT) for three days. After centrifuging at 2,500 rpm for 15 min, supernatants were removed and aseptically stored at -20°C until use.

Isolation of PBMC - Heparinised venous blood was obtained from healthy adult volunteers who had given informed consent. PBMC were isolated by density gradient centrifugation with Histopaque [density (d) = 1.077] (Sigma Chemical Co, St Louis, MO, USA). Briefly, 5 mL of heparinised blood was mixed with an equal volume of Roswell Park Memorial Institute (RPMI)-1640 tissue culture medium (Gibco Laboratories, Grand Island, NY, USA) containing 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 20 mM HEPES and 40 µg/mL gentamicin [complete tissue culture medium (CTCM)]. Samples were layered over 5 mL Histopaque in a 15 mL plastic conical centrifuge tube. After centrifuging at 300 g for 30 min at RT, the interface layer of PBMC was carefully aspirated and washed twice with PBS-ethylenediamine tetraacetic acid and once with CTCM at 300 g for 30 min. Cell viability was determined by 0.2% Trypan blue exclusion and was > 95% in all experiments. Monocytes were counted using neutral red (0.02%) and the mononuclear cells were suspended at a concentration of 2 x 106 monocytes/mL in CTCM. Monocyte preparations routinely contained > 90% monocytes, as determined by morphologic examination and staining for nonspecific esterase (Li et al. 1973).

MGC formation - Monocyte suspensions (2 x 10^6 cells/mL) were dispensed as a 50 µL droplet in the centre of a well in a 24-well flat-bottomed plate (Linbro Titer-tek, Flow Laboratories Inc, Mclean, VA) to produce a dense monolayer of adherent monocytes (Enelow et al. 1992a). After incubation for 2 h at 37°C under 5% CO₂, the wells were rinsed twice with RPMI and 1 mL CTCM was added to each well. For generation of MGC, monocytes were cultured adherently in CTCM with one of the following additions: PbAg (100 µg/mL), PbAg plus GM-CSF (50, 100, 200, 500 and 1,000 IU/mL), PbAg plus IFN-γ (50, 100, 300 and 1,000 IU/mL), PbAg plus IL-10 (50, 100 and 200 IU/mL), PbAg plus TGF-β (50, 100, 250 and 500 pg/mL), PbAg plus TNF-α (50, 100 and 200 IU/mL) or PbAg and GM-CSF with the other cytokines (IFN-γ, IL-10, TGF-β, or TNF-α). All the recombinant cytokines were purchased from R & D Systems (Minneapolis, MN, USA). The optimal concentration of these cytokines was standardised and utilised in the MGC formation assay. Cells were checked for clustering
and fusion after three days of culture using an inverted microscope (Nikon, Tokyo, Japan). At this time the culture medium was removed from the wells, cells were fixed and stained with May-Grunwald-Giemsa (Sigma).

Determination of the fusion index (FI) - The fusion rate of monocytes was determined by counting the number of stained nuclei within MGC (defined as cells with 3 or more nuclei per cell) and the total number of nuclei within a given field under a microscope at 200X magnification (Nascimento et al. 2008). The FI was calculated according to the following formula: FI (%) = (number of nuclei within MGC/total number of nuclei counted) x 100. In each experiment, between 300-500 nuclei were counted from selected representative fields.

Fungicidal activity of MGC challenged with Pb18 - Monocyte cultures for MGC generation were cultured for 72 h with PbAg and the different cytokines as described above. Supernatants were then removed and the cultures were challenged with 4 x 10^4 viable Pb18 yeast cells. After 4 h of co-culture (experimental cultures), MGC and Pb18 cells were harvested by aspiration with sterile distilled water to lyse the human cells. Each culture and wash was collected in a final volume of 2 mL; then, 0.1 mL from these suspensions were plated on plates with brain-heart infusion agar medium (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5% of gentamicin, 4% normal horse serum. Also, 5% of 192 water extracts from the P. brasiliensis strain was added as the source of growth-promoting factor (Kurita et al. 1993). As a control, a culture containing only 100 µL of viable Pb18 yeast cells (4 x 10^4 viable units/mL in complete medium) was submitted to the same procedures. Each culture had three inoculated plates triplicate, which were incubated at 35°C in sealed plastic bags to prevent drying. After 10 days, the number of colony-forming units (CFU) per plate was counted using a colony counter (Quimis, São Paulo, Brazil). The percentage of fungicidal activity was determined by the following formula: % fungicidal activity = 1 - (mean CFU of experimental culture/mean CFU of control culture) x 100.

Statistical analysis - Data were analysed using GraphPad InStat (GraphPad Software, version 3.05, San Diego, CA, 2000). Differences in FI, MGC formation and fungicidal activity of MGC cultured with or without PbAg and different cytokines were compared by one-way analysis of variance followed by multiple comparisons according to the Tukey-Kramer method. Significance was determined at p < 0.05.

RESULTS

GM-CSF enhances FI in PbAg-challenged MGC - The optimal concentration of the various cytokines to be employed in the assays for PbAg-stimulated MGC formation was determined before the beginning of the experiments. Initially, the human monocytes were cultured with or without different concentrations of IFN-γ (50, 100, 300 and 1,000 IU/mL), GM-CSF (50, 100, 200, 500 and 1,000 IU/mL), TNF-α or IL-10 (50, 100 and 200 IU/mL), or TGF-β (50, 100, 250 and 500 pg/mL) and either alone or in association with PbAg. The best effect on monocyte fusion was obtained when the cells were co-cultured with cytokines and PbAg (Fig. 1).

The modulatory effects of IFN-γ, GM-CSF, IL-10, TGF-β, and TNF-α on MGC formation from monocytes stimulated with PbAg showed that treatment with PbAg only or PbAg with GM-CSF, IFN-γ and TNF-α induced significantly higher FI than those observed in the non-stimulated control cultures (Fig. 2). When GM-CSF was added to the cultures stimulated with PbAg plus IFN-α, the fusion rate was significantly higher than cultures stimulated with only PbAg. In contrast, the addition of IL-10 or TGF-β to the cultures stimulated with PbAg led to low MGC formation and had similar formation with the control cultures. The addition of GM-CSF to cultures treated with PbAg plus IFN-γ significantly enhanced FI in PbAg-challenged MGC formation.

Fig. 1: the multinucleated giant cells fusion index was determined from human monocytes cultured for three days without stimulus (control), or with Paracoccidioides brasiliensis antigens (PbAg) (100 µg/mL), or association of PbAg with granulocyte macrophage colony-stimulating factor (GM-CSF) (100 IU/mL), interferon-gamma (IFN-γ) (300 IU/mL), interleukin (IL)-10 (50 IU/mL), transforming growth factor beta (TGF-β) (250 pg/mL) or tumour necrosis factor-alpha (TNF-α) (50 IU/mL). Results are expressed as mean ± standard error of the means of 20 healthy subjects. *: (p < 0.001) vs. control; Δ: (p < 0.05) vs. PbAg + IFN-γ, PbAg + GM-CSF, PbAg + TGF-β, PbAg + IL-10.

Fig. 2: typical examples of multinucleated giant cells formation after three days of monocyte cultured with Paracoccidioides brasiliensis antigens (PbAg) (A) (100 µg/mL) (200X) or PbAg (B) (100 µg/mL) in association with granulocyte macrophage colony-stimulating factor (100 IU/mL) (200X). C: Langhans giant cells (400X); D: foreign body giant cells (400X).
Formation of predominantly FBGC with cytokines

- After monocyte stimulation with PbAg or PbAg plus GM-CSF, IFN-γ, IL-10, TGF-β, or TNF-α, predominantly FBGC-like cells were formed with all the various stimuli (Table). In cultures stimulated with PbAg plus IFN-γ, there was a slight increase in the formation of LGC-like cells. Fig. 3 shows representative photomicrographs of MGC formation, FBGC-like and LGC-like cells.

GM-CSF upregulates fungicidal activity of MGC challenged with Pb18 - Fungal activity of MGC against Pb18 induced by PbAg with pro or anti-inflammatory cytokines can be observed in Fig. 2. MGC generated with only PbAg or PbAg plus GM-CSF or IFN-γ presented significantly higher fungicidal activity compared to control cultures, non-stimulated cultures or cultures treated with IL-10, TGF-β, or TNF-α. The highest fungicidal activity of MGC was obtained after stimulation with PbAg plus GM-CSF and IFN-γ; the lowest fungicidal activity was detected when IL-10 was added to the culture stimulated with PbAg. The addition of GM-CSF to cultures treated with IL-10 induced low fungicidal activity relative to the cultures stimulated with the other cytokines, but significantly higher than cultures treated only with IL-10. These results demonstrate that the inhibitory effect of IL-10 on the cultures was partially reverted by GM-CSF treatment and GM-CSF enhanced the capacity of MGC to kill the fungus.

**DISCUSSION**

This study demonstrated for the first time that GM-CSF is the best cytokine to induce FI of monocyte-derived MGC challenged with PbAg. Additionally, GM-CSF significantly increased the fungicidal activity of MGC against Pb18 with the addition of IFN-γ.

The antigen employed for MGC generation induced a significant increase in monocyte fusion within 72 h of culture. This cell-free-antigen (CFA) contains polypeptides in the 10–110 kDa range. The specific antigen 43 kDa (gp43) of *P. brasiliensis* predominates in all CFA preparations that are employed for the diagnosis of paracoccidioidomycosis (Camargo et al. 1991). As gp43 interacts with the mannose receptors in macrophages (Almeida et al. 1998, Popi et al. 2002), it is possible for the CFA to induce MGC formation by a mannose receptor-mediated mechanism previously suggested by DeFife et al. (1997). Other parasitic antigens have been employed in studies of MGC formation. The in vitro generation of MGC derived from PBMC of patients with active schistosomiasis stimulated with soluble egg antigen or adult worm antigen preparations from *Schistosoma mansoni* was attributed to persistent antigenic stimulation (Silva-Teixeira et al. 1993). Heat-killed *Candida albicans* yeast cells also induced monocyte proliferation and MGC formation within four days of culture (Heinemann et al. 1997). According to Gasser and Most (1999), direct contact of monocytes with *Mycobacterium bovis*, in combination with cytokines present in the supernatant cultures, is important to induce MGC formation. Additionally, membrane-bound molecules of mycobacterium and monocytes are involved in the fusion process.

Our PbAg-induced MGC formation results and the presence of yeast cells in granulomas of paracoccidioidomycosis suggest that these cells play an important role in the immunoregulatory mechanisms involved in granuloma formation against Pb18. Previous morphologic studies of histopathological lesions in human and experimental models of paracoccidioidomycosis found that granulomas consist of accumulated macrophages, epithelioid cells and Pb18 yeast cells of various sizes and sporulation inside MGC (Peraçoli et al. 1982, Montenegro & Franco 1994, Parise-Forrest & al. 2006). These MGCs may be formed by the macrophage fusion around the fungus that secretes several antigens and can chronically stimulate these cells.

The study on the modulatory effects of cytokines on human monocytes-derived MGC formation with PbAg stimulation indicates that the addition of IFN-γ or TNF-α to monocyte cultures stimulated with PbAg did not significantly enhance FI relative to cultures stimulated with only PbAg. Anti-inflammatory cytokines IL-10 and TGF-β had inhibitory effects on monocytes cell fusion and MGC formation in cultures stimulated with PbAg. Our results are consistent with other publications reporting a regulatory effect of IL-10 on the MGC generation (Ikedo et al. 1998). IL-10 may also play a role in granuloma formation during infection with *M. bovis*; animals deficient in IL-10 and infected with *M. bovis* develop a more severe granulomatous response that is responsible for the microorganism elimination (Jacobs et al. 2000).

However, the fusion rate significantly increased when GM-CSF was added to monocyte cultures stimulated with PbAg in comparison to cultures only stimulat-
ed with PbAg. Other studies demonstrated that GM-CSF acts as an induction agent for MGC formation (Lee et al. 1993, Lemaire et al. 1996). Moreover, the addition of GM-CSF to cultures stimulated with PbAg plus IFN-γ led to significantly higher fusion rates than cultures stimulated with PbAg alone or PbAg plus GM-CSF or IFN-γ. These results suggest that GM-CSF has a stimulatory effect on monocyte activation that leads to increased cell fusion when stimulated with PbAg and IFN-γ. The highest amount of MGC formation was observed in monocyte cultures stimulated with PbAg plus GM-CSF and IFN-γ. These results suggest a synergistic effect of GM-CSF on monocyte fusion induced by IFN-γ.

Evaluation of differentiated LGC and FBGC-like cell morphology after cytokine-stimulation demonstrated that, despite a slight increase in LGC formation after PbAg plus IFN-γ stimulation, there was a higher percentage of FBGC in all the cultures treated with the various combinations of cytokines. These results are contrasting to previous literature reporting a higher number of LGC with IFN-γ stimulation. One possible explanation for these differences may be related to the incubation time for monocyte-derived MGC formation. Some authors stimulate monocytes with IFN-γ for seven-14 days to obtain a high number of LGC (Most et al. 1990, Seitzer et al. 2001). In our study, monocytes were stimulated three days for MGC generation, similar to other authors (Gasser & Most 1999, Anderson 2000, Zhu & Friedland 2006). However, it is accepted that FBGC may represent LGC in early stages of formation prior to the circular arrangement of nuclei (McNally & Anderson 1995, Mizuno et al. 2001). FBGC may mature to LGC by intracellular cytoskeleton movements as previously suggested (Adams 1976).

The fungicidal activity of monocyte-derived MGC treated with viable Pb18 yeast cells was also comparatively assessed. The fungicidal activity of MGC stimulated with PbAg plus IFN-γ or PbAg plus IFN-γ and GM-CSF was significantly higher than the activity of MGC induced by TNF-α, IL-10 and TGF-β. These results confirm our previous study demonstrating that IFN-γ and PbAg promote MGC formation and also the fungicidal activity of these cells (Nascimento et al. 2008).

Although the function of MGC remains unknown, Enelow et al. (1992a) demonstrated that MGC have greater fungicidal activity against C. albicans than macrophages. The increased fungicidal activity of MGC was attributed, at least in part, to their enhanced oxidative activity. MGC are formed under inflammatory conditions and MGC are more responsive to cytokine exposure than macrophages (Enelow et al. 1992b). The elevated MGC fungicidal activity against Pb18 observed in our cultures could be explained by the enhanced activation of these cells by pro-inflammatory cytokines.

Previous studies on the fungicidal activity of human monocytes against Pb18 indicated that IFN-γ plays an important role in monocyte and macrophage activation, which leads to the inhibition of fungal replication in these cells (Calvi et al. 2003). The synergistic effects of IFN-γ and GM-CSF on MGC formation were also observed in the fungicidal activity of these cells against Pb18. GM-CSF is known to activate monocytes and promote microbicidal activity (O’Mahony et al. 2008); therefore, GM-CSF may be important to enhance fungicidal activity of MGC challenged by Pb18.

The treatment of PbAg-induced MGC with IL-10 and TGF-β significantly inhibited the fungicidal activity against Pb18. These regulatory cytokines also suppress immune responses against M. tuberculosis by inhibiting macrophage function, thus, resulting in intracellular growth of this pathogen (Jacobs et al. 2000). However, the suppressive effects of these cytokines were attenuated and enhanced fungicidal activity was observed when GM-CSF was added to PbAg-stimulated cultures and treated with IL-10 or TGF-β. These results suggest that GM-CSF reverts the inhibitory effects of IL-10 and TGF-β on MGC formation. Together our results show that GM-CSF plays an important role in PbAg-stimulated MGC formation and enhances the fungicidal activity of these cells. The positive effects of GM-CSF on fungicidal activity, even in the presence of anti-inflammatory cytokines, suggest a modulatory effect on MGC formation and fungicidal activity against Pb18.

REFERENCES


