Inhibitory effect of PGE$_2$ on the killing of Paracoccidioides brasiliensis by human monocytes can be reversed by cellular activation with cytokines

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Paracoccidioides brasiliensis is the etiological agent of paracoccidioidomycosis, a deep mycosis endemic in Latin America. Studies to elucidate the host-parasite relationship in this mycosis have demonstrated that non-activated phagocytes fail to kill the etiologic agent. Investigations of human monocytes have shown that the lack of fungicidal activity is partially associated with the capacity of a high-virulence strain to induce PGE$_2$ release by these cells. This eicosanoid inhibits production of TNF-α, the cytokine involved in cell activation for release of H$_2$O$_2$, the fungicidal metabolite. Cell priming with IFN-γ was shown to partially reverse this inhibitory effect. In this study, we asked whether monocyte challenge with a low-virulence strain of this fungus would also result in PGE$_2$ release and consequently inhibition of antifungal activities. We also assessed whether PGE$_2$, besides inhibiting production of TNF-α, a monocyte-activating cytokine, also affects IL-10. The latter, in contrast to TNF-α, is a monocyte-suppressing cytokine. Finally, we evaluated whether priming cells with other cytokines, namely TNF-α and GM-CSF, could be more effective than IFN-γ in reversing the PGE$_2$ inhibitory effect. The results revealed that the less virulent P. brasiliensis strain also induces human monocytes to release PGE$_2$. However, the inhibitory effect of PGE$_2$ was less pronounced when cells were challenged with this strain than with the more virulent one. It was also demonstrated that PGE$_2$, while inhibiting TNF-α production, tends to increase IL-10 levels. Priming with GM-CSF or TNF-α was more effective than IFN-γ in compensating for the inhibitory PGE$_2$ effect, since these cytokines induce cells to produce higher H$_2$O$_2$ and TNF-α levels.

Keywords Prostaglandin E$_2$, cytokines, monocytes, P. brasiliensis, hydrogen peroxide

Introduction

Paracoccidioidomycosis is the most prevalent deep mycosis in Latin America. The etiologic agent, Paracoccidioides brasiliensis is a thermally dimorphic fungus that develops as yeast at body temperature and as a mycelium at room temperature [1]. Since the etiological agent is considered an intracellular pathogen, studies on the mechanisms involved in its interaction with phagocytic cells are essential for elucidating the host-parasite relationship. In this context, our laboratory has been interested in recent years in studying the effect of modulatory molecules on the anti-fungal activities of human monocytes. Among these, prostaglandins (PGs) have warranted special attention. PGs are eicosanoids synthesized from arachidonic acid by a complex metabolic pathway involving the enzyme cyclooxygenase. These potent
lipidic molecules regulate key aspects of the immune response [2,3]. For instance, PGs, mainly PGE₂, act on Th0 clones to induce the synthesis of Th2 cytokines with a subsequent inhibition of Th1 response [4,5]. PGE₂ also inhibits chemokine production and lymphocyte proliferation, and has a pronounced inhibitory effect on phagocyte and dendritic cell functions [6–9]. As a function of these actions, PGE₂ liberation has been considered an important mechanism for the pathogenesis of some infectious diseases, mainly those whose resistance is controlled by macrophage activation induced through Th1 type cytokines [10,11]. It has been demonstrated studies on the role of this mediator in the pathogenesis of paracoccidioidomycosis are scarce. However, it has been demonstrated that PGE₂ mediates immunosuppression detected in early phases of murine P. brasiliensis infection by increasing IL-4 and IL-10 production [12].

P. brasiliensis strains may vary in their virulence, with Pb18 and Pb265 being considered standard strains of high and low virulence, respectively. The differences in virulence were determined by both in vitro and in vivo studies. Pb265 did not evoke specific lesions in mice, and the levels of cellular immunity were significantly lower than those found with Pb18. Lung granulomas induced by strain Pb18 had higher concentrations of fungi and neutrophils and were found with Pb18. Lung granulomas induced by strain Pb18 had higher concentrations of fungi and neutrophils and lower levels of mononuclear cells [13]. Moreover, Pb265 induced weak antibody response whereas Pb18 isolates caused stronger specific humoral responses in genetically susceptible mice [14]. The recruitment of inflammatory cells to the peritoneal cavity in rats inoculated with cell wall fraction from Pb265 was greater than that observed for the cell wall fraction recovered from the virulent strain [15]. It has also been demonstrated that Pb265 is more easily killed by phagocytes when compared to Pb18 [16,17].

Previous studies in our laboratory have demonstrated that a virulent strain of P. brasiliensis induces PGE₂ production by human monocytes, which in turn inhibits TNF-α and H₂O₂ production. As a result, cells lack fungicidal activity against the fungus since H₂O₂ is the main metabolite involved in P. brasiliensis killing. Monocyte activation with IFN-γ resulted in a slight increase in TNF-α and H₂O₂ production and consequently in fungicidal activity [16,18]. In an attempt to elucidate the modulatory effect of PGE₂ on monocyte functions against P. brasiliensis, we asked in this study whether monocyte challenge with a low-virulence strain of this fungus could also result in PGE₂ release and consequently in inhibition of monocyte antifungal activities. We also assessed whether, in addition to TNF-α, IL-10 levels were also affected by PGE₂. Finally, we evaluated whether cell activation could be more effective with other cytokines, specifically TNF-α and GM-CSF, than with IFN-γ on reversing PGE₂ inhibitory effect.

The results revealed that the less virulent P. brasiliensis strain (Pb265), similarly to the more virulent strain (Pb18), induced human monocytes to produce PGE₂. However, when monocytes were challenged with the less virulent strain, the inhibitory effect was less pronounced, probably due to the fact that Pb265 induces higher TNF-α production by the cells. It was also demonstrated that PGE₂, while simultaneously inhibits TNF-α production, tends to increase IL-10 levels. TNF-α and GM-CSF were each more effective than IFN-γ in compensating for the inhibitory effect of PGE₂, as these cytokines induce cells to produce higher H₂O₂ and TNF-α levels.

Materials and methods

Donors

Eighteen healthy blood donors from the University Hospital of the Botucatu Medical School, São Paulo State University, Brazil (age range 20–50 years) were included in this study. The Research Ethics Committee of Botucatu Medical School approved the study, and informed consent was obtained from all the blood donors.

Isolation of peripheral blood mononuclear cells

Heparinized venous blood was obtained from healthy adults. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation at 400 g for 30 min on Ficoll-Paque Plus [density (d) = 1.077] (GE Healthcare Bio-Sciences AB, Uppsala). Briefly, 20 ml of heparinized blood was mixed with an equal volume of RPMI–1640 tissue culture medium (Sigma-Aldrich, St Louis, USA), and samples were layered over 10 ml of Ficoll-Paque Plus in a 50 ml conical plastic centrifuge tube. After centrifugation at 400 g for 30 min at room temperature, the interface layer of PBMC was harvested and washed twice with RPMI-1640 tissue culture medium (Sigma-Aldrich). The PBMC suspension was stained with neutral red (0.02%) which is incorporated by monocytes to allow their identification and counting in a hemocytometer chamber. After counting, the mononuclear cell suspension was adjusted to 2 × 10⁶ monocytes/ml in RPMI-1640 (Sigma-Aldrich) containing 2 mM L-glutamine, 10% heat-inactivated human autologous serum, 20 mM HEPES and 40 μg/ml gentamicin (Complete Tissue Culture Medium – CTCM), dispensed into 96-well flat-bottomed plates at 100 μl/well (TPP, Trasadingen, Switzerland) and used for evaluation of fungicidal activity and H₂O₂ production. Another suspension was adjusted to 1 × 10⁶ monocytes/ml in CTCM and dispensed at 100 μl/well into 24-well flat-bottomed plates (TPP). After incubation of both cultures for 2 h at 37°C in 5% CO₂, nonadherent cells were removed by aspiration and each well was rinsed twice with RPMI-1640 tissue culture medium. The resulting cultures of monocytes were treated with the

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following stimuli for 18 h at 37 °C in 5% CO₂: (i) CTCM, (ii) Indomethacin (INDO) 20 μg/ml (Sigma-Aldrich), (iii) IFN-γ 50 U/ml, (iv) TNF-α 50 U/ml or (v) GM-CSF 50 U/ml (all from R&D Systems, Minneapolis, MN, USA) depending on the experiment. The viabilities of human monocytes, after different treatments, were evaluated by the reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) to purple formazan. Controls consisted of cells incubated only with RPMI. After removing the supernatants, cells in each well (three wells for each group) were incubated with 1 mg/ml of MTT in RPMI-1640 for 3 h at 37 °C and 5% CO₂. The assay was stopped by adding dimethyl sulfoxide (DMSO, 150 μl/well) (Dinâmica Química Contemporânea LTDA, SP, Brazil) for 5 min [19,20]. The formazan was quantified using a microplate reader (MD 5000, Dynatech Laboratories) to measure the absorbance at a wavelength of 540 nm. Results of cell viability were calculated by the formula = 1 − (A540 of treatment well/A540 of control well) × 100%.

Fungi

P. brasiliensis strain 18 (Pb18) and strain 265 (Pb265) held in the mycology culture collection of the Department of Microbiology and Immunology, Biosciences Institute, UNESP, were used throughout this study. Pb18 and Pb265 yeast cells were maintained by weekly subcultivation at 35 °C on 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium (GPY medium) (all reagents from Oxoid Ltd., Basingstoke, England), and yeast cells having been cultured for 6 days were taken from culture, washed and suspended in 0.15 M phosphate-buffered saline (PBS pH 7.2). In order to obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (3 cycles of 10 sec). Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted. The inoculum used for the challenge was also plated according to the same conditions. The plates containing the material obtained from the monocyte-fungus cocultures were considered the experimental plates and those plated with the inoculum alone and counted at time zero were used as controls. Fungicidal activity percentage was determined by the following formula:

\[
\text{% Fungicidal Activity} = \left[1 - \left(\frac{\text{mean CFU recovered on experimental plates}}{\text{mean CFU recovered on control plates}}\right)\right] \times 100
\]

H₂O₂ release

The release of H₂O₂ by monocytes was measured by the horseradish peroxidase-phenol red oxidation method [23,24]. Following treatments, supernatants were removed and monocyte cultures containing 2 × 10⁶ cells/ml were challenged or not with 100 μl of a Pb18 or Pb265 suspension containing 4 × 10⁴ viable yeast cells/ml (fungus-to-monocyte ratio of 1:50) prepared in phenol red buffer containing 50 μl horseradish peroxidase type II (Sigma Chemical Co) and 10% fresh autologous serum, incubated for 4 h at 37°C in 5% CO₂. The reaction was stopped by the addition of 10 μl of 1N NaOH, and the absorbance at 620 nm was determined with a micro-ELISA reader (MD 5000; Dynatech Laboratories Inc., Chantilly, VA, USA). All the determinations were done in triplicate, and the absorbance was transformed into nanomoles based on a standard curve serially diluted from 0.5 to 8 nanomoles H₂O₂.

TNF-α, IL-10 and PGE₂ determination

Following treatments, supernatants were removed and monocyte cultures containing 1 × 10⁶ cells/ml were challenged with 1 ml of a Pb18 or Pb265 suspension containing 2 × 10⁴ viable yeast cells/ml (fungus-to-monocyte ratio of 1:50) prepared in CTCM plus 10% fresh autologous serum or CTCM alone, for 4 h at 37°C in 5% CO₂. After this period, supernatants were collected, separated from cell debris by centrifugation at 1000 g for 15 min, and stored under the same conditions described above. Next, cocultures were harvested by aspiration with sterile distilled water to lyse monocytes. Each well washing resulted in a final volume of 2.0 ml and 0.1 ml was plated on supplemented brain-heart infusion (BHI) agar medium (Oxoid) containing 0.5% gentamycin, 4% horse normal serum and 5% P. brasiliensis strain 192 culture filtrate (v/v), the latter being the source of growth-promoting factor [22]. Inoculated plates, in triplicate from each culture, 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. The inoculum used for the challenge was also plated according to the same conditions. The plates containing the material obtained from the monocyte-fungus cocultures were considered the experimental plates and those plated with the inoculum alone and counted at time zero were used as controls. Fungicidal activity percentage was determined by the following formula:

\[
\text{TNF-α, IL-10 and PGE}_2 \text{ determination}
\]

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Reversing the inhibitory effect of PGE$_2$

Statistical analysis

Data were analyzed statistically using the INSTAT software (Graph Pad, San Diego, CA). The results were compared by analysis of variance (ANOVA) followed by the Tukey test, with the significance level set at $P < 0.05$.

Results

**PGE$_2$ production by human monocytes challenged with Pb18 or Pb265**

Our first objective was to compare the capacity of two *P. brasiliensis* strains to induce PGE$_2$ release by human monocytes (Fig. 1). However, as previous studies in our laboratory have shown that the fungus itself produces prostaglandins, cultures of Pb18 and Pb265 alone were also evaluated. Non-challenged monocytes produced low PGE$_2$ levels, which were significantly increased after *P. brasiliensis* challenge. Interestingly, there were no significant differences in the PGE$_2$ levels induced by the two strains. Pb18 and Pb265 alone also produced PGE2, the concentration of which was similar between them, but significantly different from those released by challenged cells. Thus, the vast majority of PGE2 levels were released by monocytes challenged by the fungus rather than by the fungus itself. INDO, a cyclooxygenase inhibitor, significantly inhibited PGE$_2$ production in all co-cultures (Fig. 1). The inhibitory effect of INDO treatment on PGE$_2$ production by human monocytes was not due to the influence of INDO on cell viability since the MTT assay demonstrated that the metabolic activity of these cells remained $\approx 100\%$ when compared with the control cultures treated with only RPMI.

**Inhibition of fungicidal activity by PGE$_2$ is more pronounced in response to Pb 18**

Given that INDO significantly inhibited PGE2 production, we decided to use this pharmacological approach to study the effect of this eicosanoid on the antifungal activity of monocytes. First, the effects on fungicidal activity against the two strains were evaluated (Fig. 2). Non-treated monocytes showed low fungicidal activity against both strains of *P. brasiliensis*. However, this activity was significantly elevated when cells were treated with INDO before being challenged by the fungi. Interestingly, higher fungicidal action was detected in response to Pb 265. Thus, although both strains induce cells to release equivalent concentrations of PGE$_2$, the inhibitory effect of INDO on PGE$_2$ release is more pronounced for Pb18.
of PGE₂, inhibition was higher in response to Pb18. To confirm the inhibitory effect of PGE₂ in the killing of \textit{P. brasiliensis} by human monocytes, the co-cultures were treated with exogenous PGE₂ and simultaneously challenged with Pb18 or Pb265. The results demonstrated that PGE₂ treatment significantly inhibited the fungicidal activity of human monocytes against the two fungus strains, mainly at concentrations of 625 and 1250 pg/ml (Fig. 3).

\textit{PGE₂} inhibits \(H₂O₂\) release by human monocytes challenged with Pb18 or Pb265

In an attempt to elucidate the inhibitory effect of PGE₂ on the fungicidal activity of human monocytes against Pb18 and Pb265, \(H₂O₂\) production was evaluated in all cultures as it is the metabolite involved in fungus killing by monocytes (Fig. 4). Control monocytes (without INDO treatment and non-challenged) released substantial \(H₂O₂\) levels, which tended to be lower after challenge with Pb18 or Pb265. After INDO treatment, the levels were significantly increased in all cultures, reinforcing the inhibitory role of PGE₂ in producing this metabolite. However, it should be noted that this inhibition was more pronounced in response to Pb 18, given that \(H₂O₂\) production after challenge with Pb265 was significantly higher in comparison with Pb18.

\textit{PGE₂} inhibits TNF-\(\alpha\), but increases IL-10 production by human monocytes challenged with Pb18 or Pb265

Our next question was to evaluate whether monocyte inhibition by PGE₂ was associated with alterations in the levels of cytokines classically involved in activation or suppression of monocyte/macrophage activities. Thus, TNF-\(\alpha\) and IL-10 levels were evaluated in supernatant co-cultures. As can be seen in Fig. 5, higher TNF-\(\alpha\) levels were detected after challenge, mainly in response to Pb18. However, these levels were significantly increased after INDO treatment, showing the inhibitory effect of PGE₂ in producing this cytokine. IL-10 production also tended to be increased after challenge, especially with Pb18 (Fig. 6). However, in contrast to the TNF quantity detected, the levels tended to
be reduced after INDO treatment, indicating that PGE2 may increase IL-10 production by human monocytes.

Monocyte activation with TNF-α or GM-CSF is more effective than IFN-γ in increasing fungicidal activity by human monocytes

Given the observations that PGE2 is released by human monocytes in response to a P. brasiliensis strain of either lesser or greater virulence and that eicosanoid inhibits antifungal activities of these cells, we hypothesized that these effects could be reversed by monocyte priming by activating cytokines. In previous studies we have shown that IFN-γ activation resulted in a discrete increase in antifungal activities against virulent P. brasiliensis strain [16,18]. Thus, in the present study we asked whether priming cells with other cytokines, specifically TNF-α and GM-CSF, could be more effective than IFN-γ in reversing the inhibitory effect of PGE2.

The results for the fungicidal activity are shown in Fig. 7. Activation by TNF-α and especially GM-CSF resulted in higher fungicidal activity when compared to priming by IFN-γ. However, we reemphasize that the results produced by the strain 265 were higher than those obtained from strain 18.

Monocytes are activated more effectively by TNF-α or GM-CSF than by IFN-γ in increasing H2O2 release by human monocytes

To reinforce our idea that the activation process could reverse the inhibitory effect of PGE2, we evaluated H2O2 release after cell activation with the three cytokines. As expected, H2O2 levels were significantly increased after TNF-α and GM-CSF activation when compared with IFN-γ. Higher levels were also obtained by cells challenged with Pb265 (Fig. 8).
Cytokine activation is associated with alterations in TNF-α and IL-10 levels

TNF-α and IL-10 levels were also evaluated in culture supernatants from activated cells (Fig. 9 and Fig. 10 respectively). IFN-γ and, to a greater extent, GM-CSF-activated cells released higher TNF-α concentrations in relation to non-activated ones. Once again, TNF-α levels were higher in cultures challenged with Pb265. IL-10 production increased after cell activation with IFN-γ, TNF-α or GM-CSF. However, cytokine levels in cultures challenged with Pb265 tended to be smaller than those produced by the Pb18 strain.

Discussion

In paracoccidioidomycosis, the role of PGE2 in immune response modulation is poorly understood. In this context our group has studied in recent years the modulatory effect of these mediators on the response of phagocytes against the fungus. In this study, one result draws attention and confirms previous findings showing that the fungus itself can produce prostaglandins. However, the concentrations of fungal PGE2 are quite low when compared to those released by cells in response to the fungus. So, we are considering that the effects detected in the present study must be mainly attributed to the PGs produced by monocytes. However, we cannot definitely rule out that fungal PGE2, even at low concentrations, may also play a role. Studies in our laboratory are being carried out to characterize and purify fungal PGE2, as well as to evaluate its effects on phagocytic and other cells of the immune system.

PGE2 inhibited fungicidal activity of human monocytes against both high- and low-virulent strains of P. brasiliensis. The results also showed an association between this inhibitory process and declines in TNF-α and H2O2 production but a tendency toward a PGE2-induced increase of IL-10 levels. This modulatory effect of PGE2 on IL-10 production is reported by other studies using both human and murine cells [25–28]. Niho et al. [29], studying the role of IL-10 in the cross-regulation of PGs and cytokines in human monocytes, demonstrated that these cells, in an initial step after LPS stimulation, produce both TNF-α and PGE2. This PGE2 production may be stimulated, at least in part, by TNF-α through a mechanism dependent on COX-2 expression increase. Subsequently, PGs inhibited TNF-α release and induced IL-10 synthesis, demonstrating that the suppressor PGE2 effects could be mediated by this cytokine. According to the authors, TNF-α could also directly induce IL-10 synthesis by a mechanism independent of PGE2. Thus, at a later step, these higher IL-10 levels might inhibit not only TNF-α and PGE2 synthesis but also its own synthesis.

PGE2 induction by P. brasiliensis may be considered a means by which this fungus escapes from the effectors mechanisms of phagocytes. Such escape from oxidative metabolism has been reported in other microorganisms – including Leishmania donovani [30], Histoplasma capsulatum [31] and Mycobacterium leprae [32] – which did not induce or inhibit the production of O2 metabolites. Moreover,
some authors have demonstrated an association between the failure of certain microorganisms in triggering oxidative burst and releasing products from arachidonic acid metabolism, including PGE$_2$ [33].

Our results clearly showed that the more virulent strain provoked a greater inhibitory process than the less virulent strain, since it was demonstrated that although both strains induced similar PGE$_2$ concentrations, fungicidal activity against Pb265 was ever higher in relation to Pb18. This difference may be attributed to the ability of Pb265 to induce higher TNF-α levels, but lower IL-10 release in relation to Pb18, as demonstrated in our experiments. Higher induction of TNF-α production by Pb265 was also reported in other studies and has been attributed to the β-glucan content of its cell wall [15,17,34,35]. Therefore, we can hypothesize that despite the inhibitory effect of PGE$_2$, higher cell activation by Pb265, via TNF-α, results in an increase in H$_2$O$_2$ production and consequently, in higher fungicidal activity.

The present study also reports new findings in relation to the compensatory effect on PGE$_2$ inhibition induced by cell priming with cytokines. The results clearly showed that TNF-α and GM-CSF were more effective than IFN-γ in this process. In an attempt to clarify the mechanisms involved in these differences, H$_2$O$_2$ production was compared in control cultures and in those activated with cytokines. As expected, H$_2$O$_2$ production was higher after cell activation with TNF-α and especially with GM-CSF, when compared with IFN-γ. Consistent with the increased H$_2$O$_2$ production, TNF-α levels tend to be higher in GM-CSF activated cultures. However, it is noteworthy that in cultures primed with TNF-α, the levels of the same cytokine were lower than those detected in cultures primed with IFN-γ and despite this, H$_2$O$_2$ levels were similar to those detected in cultures primed with GM-CSF. The probable explanation is that during priming with TNF-α, cells have been directly activated with optimal concentrations of this cytokine, for the production of H$_2$O$_2$.

It should be noted that cellular activation with the cytokines resulted in higher IL-10 release. Previous studies have demonstrated that IL-10 suppresses fungicidal activity against _Paracoccidioides brasiliensis_ by inhibiting H$_2$O$_2$ production [36,37]. Thus, according to these findings we could speculate that the same mechanisms could be occurring in the present study. However, since the metabolite release was increased after activation with cytokines, we can argue that the stimulatory effects of TNF-α, whose levels also increased as a consequence of cell activation, are overlapping to the inhibitory effects of IL-10.

From these results we can strongly suggest that TNF-α is the cytokine responsible to activate signaling pathways that result in H$_2$O$_2$ production by human monocytes in response to _P. brasiliensis_. However, this fungus is able to escape from this effector mechanism by inducing cells to produce PGE$_2$, which in turn induces IL-10 production that inhibits TNF-α levels. This process is less pronounced when cells were challenged with a low-virulence _P. brasiliensis_ strain, due to its capacity to induce higher TNF-α release. This escape mechanism could be restored if host cells are adequately activated with cytokines, a process in which TNF-α and GM-CSF are more effective than IFN-γ. According to these results, we can suppose that fungicidal activity by human phagocytes in _vivo_ could be highly affected, since several studies have demonstrated that paracoccidioidomycosis patients, mainly those with an acute form of the disease, present an evident depression of mechanisms that depend on phagocyte activation [38].

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### Declaration of interest:

The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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