

Neutrophil oxidative metabolism and killing of *P. brasiliensis* after air pouch infection of susceptible and resistant mice

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Abstract: The oxidative burst of polymorphonuclear neutrophils (PMN) and their ability to inhibit *Paracoccidioides brasiliensis* growth was studied in susceptible (B10.A) and resistant (A/J) mice. The cells were obtained after subcutaneous inoculation in air pouches, yielding highly pure PMN preparations; the number of cells was similar for both strains at 24 h and five times higher in the resistant strain at 15 days. The oxidative metabolism of these PMN was evaluated by the luminol and lucigen-enhanced chemiluminescence upon stimulation with PMA or killed *P. brasiliensis* (Pb). At 24 h of infection PMN from both strains showed similar responses. However, at 15 days a great enhancement of the Pb-stimulated luminol-enhanced chemiluminescence was observed only in PMN from resistant mice. Such increase was markedly inhibited by the addition of catalase. Independent of the mouse strain or time of infection the lucigenin-enhanced chemiluminescence showed the same intensity. The lucigenin-enhanced chemiluminescence of PMN without stimuli from resistant mice did not change with the time of infection, however, after 15 days of infection a significantly lower chemiluminescence was detected with PMN from susceptible mice. At 15 days of infection the PMN from B10.A were unable to kill *P. brasiliensis* yeast cells in vitro. Because the lucigenin- and luminol-enhanced chemiluminescence detects, respectively, the O₂⁻ production and the myeloperoxidase/hydrogen peroxide halide system, the present data show parallels between deficiency in the production of oxygen-reactive species by PMN and lower fungicidal activity. *J. Leukoc. Biol.* 59: 526–533; 1996.

Key Words: polymorphonuclear neutrophils · myeloperoxidase · luminol · lucigenin · chemiluminescence

INTRODUCTION

Paracoccidioides brasiliensis is a dimorphic fungus that causes a deep human granulomatous mycosis, endemic in Latin America and frequently diagnosed in Brasil [1].

Studies using isogenic mice in experimental paracoccidioidomycosis were developed for the first time by Calich et al. in 1985, who demonstrated the existence of susceptible (B10.A) and resistant (A/Sn, A/J) mice, after intraperitoneal (i.p.) infection with a virulent *P. brasiliensis* (Pb18) isolate [2]. Although a polar behavior could be observed after i.p. infection of A/J and B10.A strains, the same was not true when the subcutaneous (s.c.) route was used; this route was unable to induce systemic disease and caused only a localized inflammatory process that progressed to cure in both A/Sn and B10.A strains [2]. An acute inflammatory infiltrate in which polymorphonuclear neutrophil (PMN) cells predominated was observed by using the s.c. and i.p. routes of infection in Swiss [3] and B10.A [4] mice, respectively. The PMN influx was previously shown to be independent of the activation of the complement system as well as of the effects of other chemotactic substances, such as prostaglandin. However, it was induced by a low-molecular-weight peptide produced by macrophages incubated with *P. brasiliensis* yeasts [4].

The role of PMN in paracoccidioidomycosis is not well established yet, as reviewed by Brummer et al. [5]. In this regard, PMN from blood of patients with paracoccidioidomycosis [6] or from peritoneal exudate of sensitized mice [7] could kill *P. brasiliensis* in vitro and inflammatory PMN had more pronounced fungicidal activity than peripheral blood PMN [7–9]. Gohman-Yahr et al. [10] showed that PMN from patients with paracoccidioidomycosis had normal phagocytic activity but had a defect in their in vitro digestive capability against phagocytosed *P. brasiliensis* yeasts when compared with PMN from healthy subjects or patients with other diseases. However, Schaffner et al. [11] have shown that PMN isolated from blood of healthy individuals ingested virulent dimorphic yeasts, triggering the

Abbreviations: CFU, colony-forming units; CL, chemiluminescence; H₂O₂, hydrogen peroxide; MPO, myeloperoxidase; O₂⁻, superoxide anion; Pb, heat-killed *Paracoccidioides brasiliensis*; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.

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respiratory burst, but were not able to kill the virulent *P. brasiliensis* yeast cells.

Substantial amounts of reactive oxygen species (ROS) are produced during phagocytosis or upon perturbation of PMN membranes by a variety of agents, such as phorbol myristate acetate (PMA) or stimuli by particulate agents. Species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO\cdot$), hypochloride ion (OCl^-), hypochlorous acid ($HOCl$), and chloramines are released from PMN through the NADPH oxidase and hydrogen peroxide-myeloperoxidase-halide systems (H_2O_2 -MPO-halide). These species released by PMN or by a cell-free system were shown to be fungicidal for dimorphic fungi such as *P. brasiliensis* [7, 12–16], and for opportunistic fungi [11, 17–19].

This activation, caused by a variety of stimuli to trigger PMN ROS production, can be determined by measuring chemiluminescence (CL) in the presence of the luminescent probes luminol and lucigenin. Products of the H_2O_2 -MPO-halide system can be measured by luminol-enhanced CL [20, 21] while superoxide anion can be detected by lucigenin-enhanced CL [21, 22].

In the isogenic murine model of paracoccidioidomycosis, it was shown that the macrophages of resistant mice are

more activated than those of susceptible ones by various parameters, such as H_2O_2 production, spreading ability, and expression of class II molecules [2]. In the present work we intended to verify whether similar differences in activation also occur in PMN of susceptible and resistant mice. Therefore we investigated the changes in the ROS production by the NADPH oxidase- and MPO-dependent systems in PMN obtained from air pouches of A/J and B10.A mice infected with a virulent *P. brasiliensis* isolate.

Previous works have demonstrated that PMN are associated with degenerated *P. brasiliensis* yeast cells in the lesions of animals in which the infection evolves to cure [23]. Therefore, in the present study, the PMN were also analyzed regarding their fungal killing ability, attempting to determine whether a correlation between higher activation and a more pronounced killing ability of PMN occurs in this experimental model.

MATERIALS AND METHODS

Animals

Male A/J and B10.A, 10- to 12-week-old mice, obtained from the Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, animal facilities were used throughout this work. The mice were fed with laboratory chow (Nuvilab CR-1, Nuvital) and acidified water ad libitum, and kept in a temperature-controlled room (22°C), housed in groups of 8–10.

Fungi

The virulent Pb18 *P. brasiliensis* isolate was maintained in potato-agar and covered with mineral oil until being employed in the experimental studies to avoid excessive subcultivation. Virulence of Pb18 was controlled by LD50% determinations and at any evidence of virulence loss the sample was re-isolated from susceptible mice according to Kashino et al. [24]. *P. brasiliensis* in its yeast form was cultivated in semi-solid Fava Netto's medium at 35°C [25] and employed on the 7th day of culture, which corresponds to the exponential phase of growth [26].

P. brasiliensis inoculation

The yeast cells were washed three times in sterile saline and the fungal suspensions were adjusted, after counting in a hemacytometer, to the desired concentration with phosphate-buffered saline (PBS). The viability of the fungal cells was evaluated using the vital dye Janus Green B (Merck) [27] and was always >85%.

Air pouches were produced on the dorsal region of mice by s.c. injection of 2 ml of air, employing the technique of Harmsen et al. with some modifications [28]. Fungal suspensions of 50×10^6 yeasts/ml in 100 μ l of PBS or 100 μ l of PBS alone were injected in the experimental groups and in the controls, respectively.

Collection of PMN

PMN were collected after 24 h and after 15 days of fungal infection. For each experiment, mice were anesthetized with ethyl ether and inoculated in the air pouches with a solution of PBS (10 mM; Merck), pH 7.4, containing 10 μ l $CaCl_2$ (1mM; Merck), 10 μ l $MgCl_2$ (0.5 mM; Merck), and 10 μ l glucose (1 mg/ml; Merck). After incision of the skin, cells were collected with a siliconized Pasteur pipette and counted in Neubauer chambers. Each air pouch yielded ~1 ml of exudate. Viability was estimated by Trypan blue exclusion and was 86% at 24 h after infection for PMN of either strain. At 15 days of infection, the viability

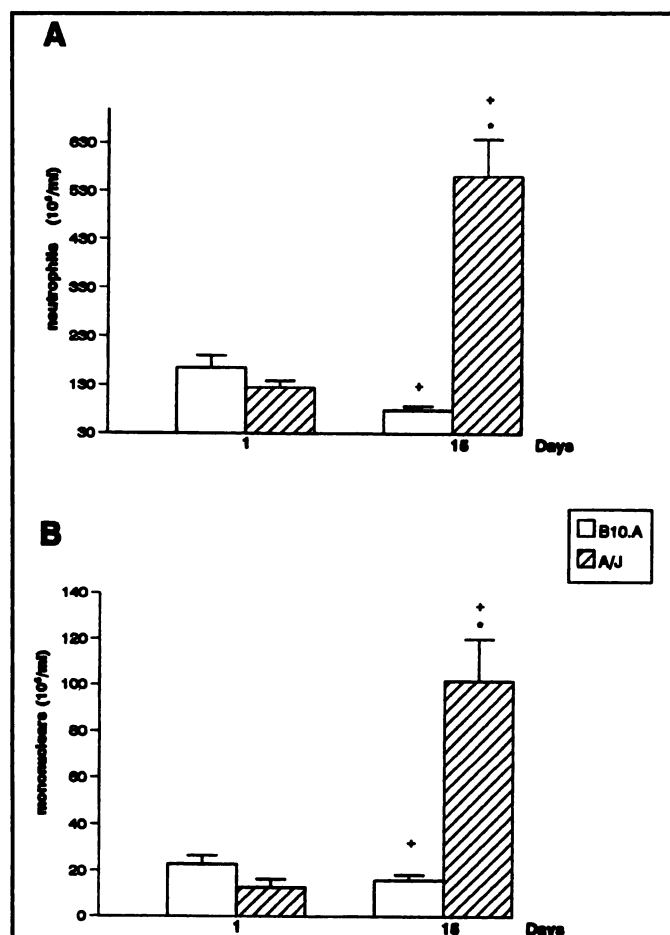


Fig. 1. Absolute counts of PMN (A) and mononuclear cells (B) from air pouches of A/J and B10.A mice, 24 h and 15 days after infection with *P. brasiliensis*. *Statistical difference between mouse strains (A/J and B10.A).

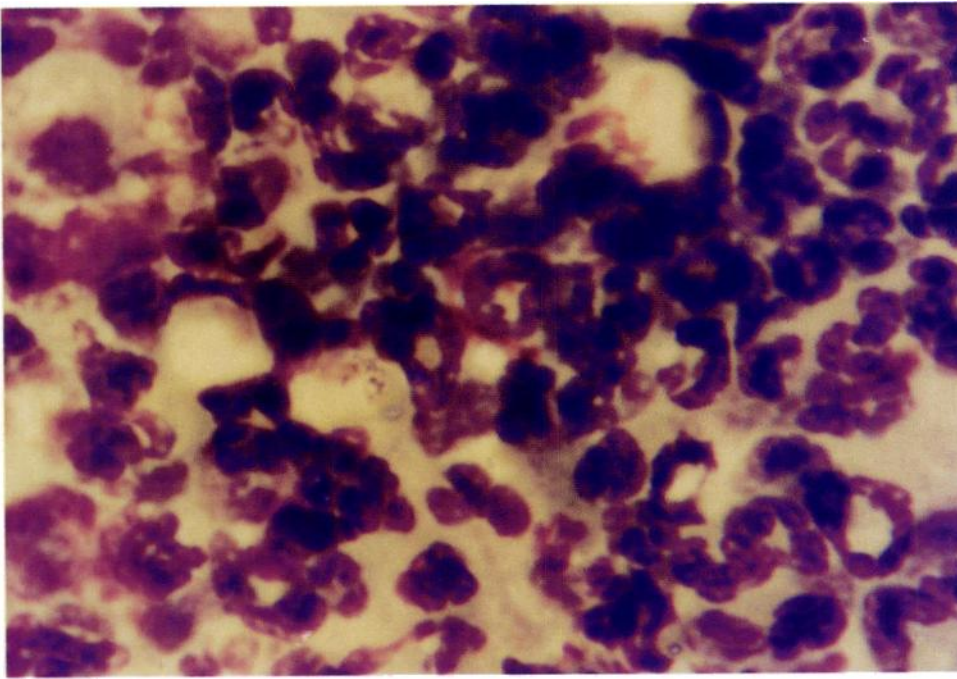


Fig. 2. Typical aspect of air pouch exudate with 81–90% of PMN cells from B10.A mice 15 days after infection with *P. brasiliensis* stained with May-Grunwald-Giemsa ($\times 1000$).

observed for PMN from A/J mice was 50% and for PMN from B10.A was 60%. The same numbers of viable PMN were always used for both strains. Cytological analyses were done in cell preparations obtained with Suta chambers and were stained with May Grunwald-Giemsa (Merck). For each assay, four to eight pools (3 animals/pool) were used.

Histopathological analysis

At 15 days after the inoculation of *P. brasiliensis* in the s.c. air pouch the mice were killed and the whole air pouch, with the formed lesion, was collected and fixed in Millonig [29], at pH 7.0–7.4. The samples were embedded in parafin and the slides stained with hematoxylin/eosin.

Chemiluminescence assays

The modified luminol-enhanced CL method of Allen and Loose [20] was used to detect the products of the oxidative metabolism dependent on the H_2O_2 -MPO-halide system. The products of the NADPH oxidase system, primarily $O_2^{\cdot -}$, were measured by the lucigenin-enhanced CL method of Mikenberg et al. [22]. In vitro stimulation was performed either with PMA or with heat-killed *P. brasiliensis* yeast cells (Pb) by steam autoclaving at $100^\circ C$, at 120 atm, for 20 min.

The reaction contained viable 1×10^6 PMN, $10 \mu l$ PMA (50 ng/ml; Sigma) or 1×10^6 killed Pb yeasts, $10 \mu l$ luminol (1 mM; Sigma) or $50 \mu l$ lucigenin (25 mM; Sigma) in 2 ml PBS. In some experiments, 200 μl of catalase (3 mg/ml; Sigma) was added to the reaction with luminol to

consume H_2O_2 . The experiments were done at room temperature (20 – $22^\circ C$) and photon emission was detected in a scintillation counter (model 1900TR-Packard, Canberra Co.). The kinetics studies were started without in vitro stimulus or by addition of stimulus, and light emission (counts per minute) was recorded during 40–60 min. Controls of CL by dead PMN and Pb showed negligible values.

P. brasiliensis killing assay by air pouch PMN

A pool of 10 mice of each strain was used to obtain PMN from air pouches after 15 days of infection and the killing assay was performed according to Mc Ewen et al. [7] with some modifications. *P. brasiliensis* yeast suspensions were obtained as previously described, the concentration adjusted to 1×10^5 yeasts/ml in Dulbecco's modified Eagle's medium (DMEM) supplemented with 30% fresh normal mouse serum. Opsonization with fresh mouse normal serum was performed during 20 min at $37^\circ C$. Mice were killed for PMN collection and the cells from the air pouch lesions were incubated in DMEM (Sigma) with 10% fetal calf serum (Cultilab). Elimination of adherent cells, mostly mononuclear cells, was performed by incubation of cell suspensions in plastic Petri dishes for 30 min at $35^\circ C$ in a 5% CO_2 atmosphere. The nonadherent cells, constituted by 95% of PMN, were collected, washed with DMEM, and adjusted to a final concentration of 5×10^6 viable PMN/ml. Cocultures were done in a ratio of 50 PMN: 1 *P. brasiliensis* yeast. The PMN suspensions (100 μl) and 100 μl of 10^4 fungi were dispensed in quadruplicate into microplate wells (Costar). Controls consisted of PMN recovered from air pouch-infected mice without in vitro addition of *P.*

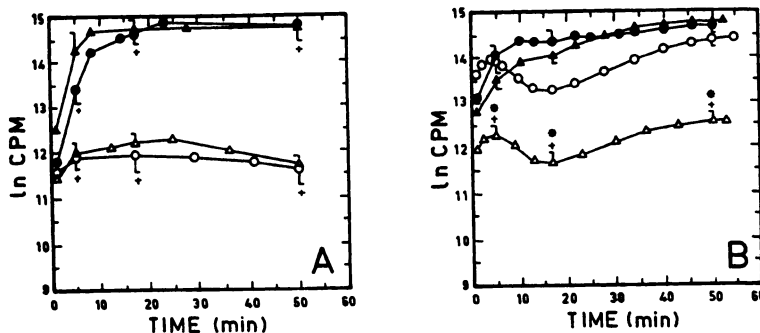


Fig. 3. Luminol-enhanced chemiluminescence of PMN from B10.A (Δ , \blacktriangle) and A/J (\circ , \bullet) obtained 24 h (A) and 15 days (B) after air pouch infection with Pb18 *P. brasiliensis*. PMN (5×10^5 /ml) were stimulated in vitro by PMA (filled symbols) or by Pb (5×10^5 /ml; open symbols). $^+$ Statistical difference between stimuli (PMA and Pb); * statistical difference between mouse strains (A/J and B10.A).

brasiliensis yeasts or of 100 μ l of 10^4 fungi cultured only in medium (DMEM + Pb). After incubation for 2 h at 37°C, in 5% CO₂, the cells were collected, the wells washed three times with 100 μ l of distilled water, and the volume of each sample was completed to 5 ml with distilled water for PMN lysis and release of ingested *P. brasiliensis* yeasts.

Volumes of 100 μ l of each sample were seeded in duplicate in plates containing brain heart infusion medium supplemented with 4% (v/v) horse serum and 5% *P. brasiliensis* isolate Pb192 culture filtrate, the latter constituting a source of growth-promoting factor [30, 31]. The plates were sealed, incubated at 37°C, and colonies were counted daily for 15 days. The results were expressed as average means and standard errors of the means of the colony forming units (CFU) counted.

Statistics

Means and standard errors of means (SE) of light emission data were transformed in neperian logarithms while means and SE of CFU counts, PMN, and mononuclear absolute counts were transformed in square root and analyzed by the Kruskal-Wallis variance tests. CL comparisons were done at 5, 17, and 30 or 50 min. The significance level considered was $P < 0.05$. Multiple comparisons were done according to Tukey [32]. For all statistical tests, the Statgraphics, V.2.6 (1987), software was used.

RESULTS

Cytological analysis

The air pouches of A/J and B10.A strains of mice had $1.2\text{--}1.6 \times 10^7$ neutrophils/ml at 24 h after *P. brasiliensis* infection. At 15 days of infection, the number of neutrophils observed in A/J mice increased five times, whereas B10.A showed a 50% decrease compared with 24 h infection (Fig. 1A). In differential counts, 81–90% of the cells were PMN and the remaining were mononuclear cells, in-

dependent of time of infection. At 24 h of infection, the absolute counts of mononuclear cells were the same (Fig. 1B). At 15 days of infection, however, the absolute number of mononuclear cells was approximately five times higher in the A/J strain than in the B10.A (Fig. 1B). Figure 2 shows a typical aspect of the exudate obtained 15 days after B10.A infection, which did not differ from that obtained with A/J mice. In control animals 10^4 neutrophils/ml were found at both analyzed periods.

Histopathological analysis

Optical microscopy observation revealed different patterns of cellular influx in the two mouse strains. The resistant A/J strain developed a typical abscess, constituted mostly by PMN and present in the lesion of typical mononuclear cells. On the other hand, the susceptible B10.A mice developed smaller lesions, with numerous PMN aggregates surrounded by mononuclear cells such as macrophages and epithelioid cells (data not shown).

Luminol-enhanced CL

Figure 3 shows the luminol-enhanced CL developed by neutrophils from A/J and B10.A mice, obtained after 24 h and 15 days of infection using PMA or Pb as stimulus. With PMA the kinetics and intensities of CL were equivalent, independent of the mouse strain or of the time of infection. The intensities of CL, as well the kinetics obtained, were dependent on the stimulus employed. PMA stimulation elicited ~16 times more CL than Pb with PMN obtained after 24 h of infection (Fig. 3A). Pb stimulation showed a biphasic kinetics typical of particulate stimulus in both strains only at 15 days of infection (Fig. 3B). At

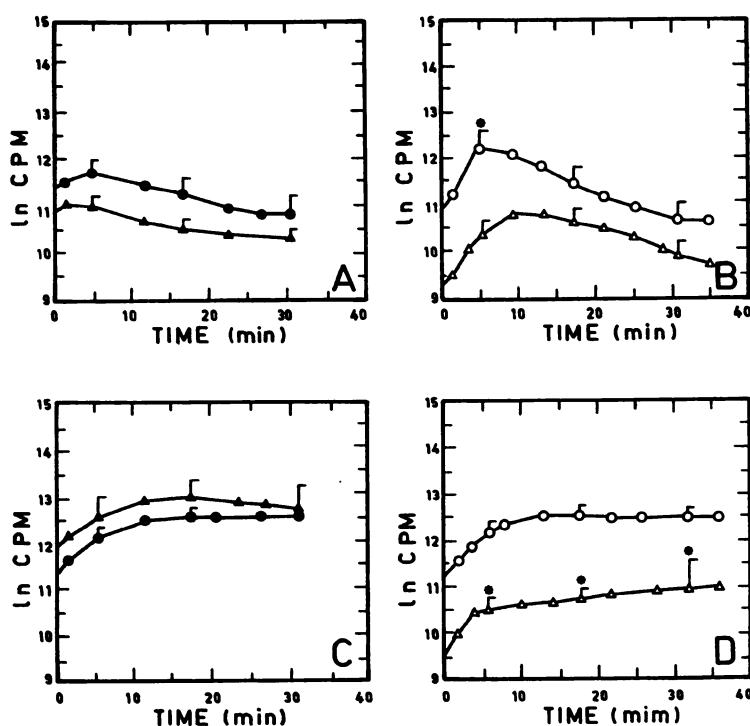


Fig. 4. Luminol (A, B)- and lucigenin (C, D)-enhanced chemiluminescence, without in vitro stimuli, of PMN from B10.A (Δ , \blacktriangle) and A/J (\circ , \bullet) obtained 24 h (filled symbols) or 15 days (open symbols) after air pouch infection with Pb18 *P. brasiliensis*. +Statistical difference between time of infection (24 h and 15 days); *statistical differences between mouse strains (A/J and B10.A).

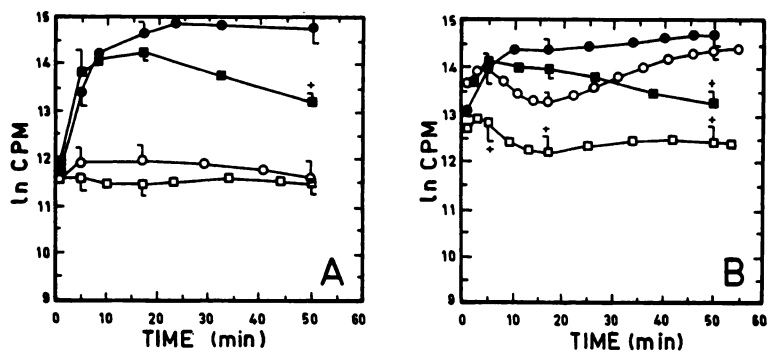


Fig. 5. Effect of catalase in the luminol-enhanced chemiluminescence of PMN from A/J stimulated by PMA (filled symbols) or by Pb (open symbols) 24 h (A) or 15 days (B) after air pouch infection with Pb18 *P. brasiliensis*. Without catalase (O, ●), with catalase (□, ■); + statistical difference between treatments.

this time of infection, a sharp difference of Pb-induced CL was seen between A/J and B10.A cells. The luminol-enhanced CL of PMN from A/J was five times higher than that of B10.A and seven times more than that of PMN collected from A/J at 24 h after infection. In contrast, no differences in CL intensities were observed for PMN from B10.A animals at any post-infection time (Fig. 3, A and B).

CL was also followed without in vitro addition of stimulus (Fig. 4). In this case, the CL intensity was lower than that obtained when PMA or Pb were added. No differences were observed after 24 h of infection for both mouse strains (Fig. 4A). However, after 15 days of infection, the luminol-enhanced CL of cells from A/J mice was approximately five times higher than that of cells from B10.A at 5 min of reaction (Fig. 4B).

Differences between animal strains and time of infection regarding the H_2O_2 -MPO-halide system could also be observed in the experiments in which catalase was added (Figs. 5 and 6). Catalase inhibited the intensity of luminol CL of PMA-stimulated PMN from both strains at both times of infection. The catalase effect was time dependent, with an inhibition of 60–80% at 50 min of reaction, respectively, for PMN obtained at 24 h and at 15 days of infection. On the other hand, catalase had no effect on Pb-stimulated PMN from both strains recovered after 24 h (Figs. 5A and 6A). The main difference between strains was observed in cells obtained after 15 days of infection, using Pb as stimulus. Catalase had no effect on B10.A cells, whereas it continuously decreased the CL of A/J mouse PMN by 65% at 5 min to 85% at 50 min (Fig. 5B).

Lucigenin-enhanced CL

Figure 7A shows the lucigenin-enhanced CL developed by A/J and B10.A neutrophils, obtained 24 h after infection, with both stimuli. The curves of PMA-stimulated PMN were essentially the same for A/J or B10.A cells at both infection times (Fig. 7, A and B). At 5 min of incubation, the Pb-stimulated A/J cells showed higher CL than those of B10.A, independent of the infection time (Fig. 7, A and B). At 15 days after infection, the CL of Pb-stimulated PMN obtained from both mouse strains showed continuous increase with incubation time (Fig. 7B).

Lucigenin-enhanced CL, without in vitro stimulation, did not show differences between the two inbred mouse strains after 24 h of infection (Fig. 4C). However, a sharp decrease in B10.A cell CL was observed after 15 days of infection (Fig. 4D).

P. brasiliensis killing by PMN

Figure 8 shows the CFU counts of viable *P. brasiliensis* recovered from exudate of air pouches from A/J and B10.A mice, at 15 days after infection, CFU of fungi cultured only in medium (100% of growth), and CFU of co-cultives of virulent *P. brasiliensis* yeasts and PMN from both mice. The number of CFU was significantly reduced (61%) by the interaction of virulent *P. brasiliensis* with A/J mouse PMN, whereas a reduction of only 14% was caused by

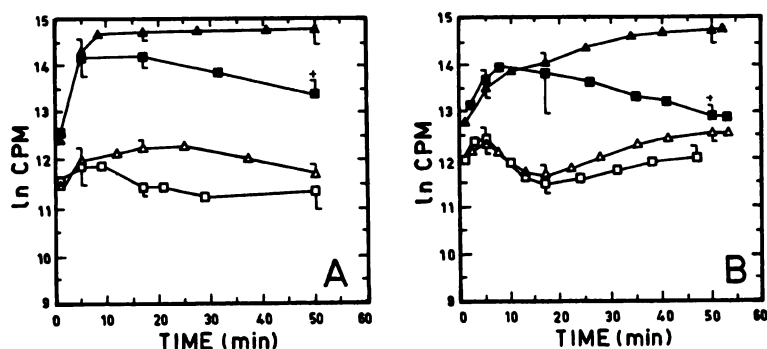


Fig. 6. Effect of catalase in the luminol-enhanced chemiluminescence of PMN from B10.A stimulated by PMA (filled symbols) or Pb (open symbols) 24 h (A) or 15 days (B) after air pouch infection with Pb18 *P. brasiliensis*. Without catalase (Δ, ▲), with catalase (□, ■); + statistical difference between treatments.

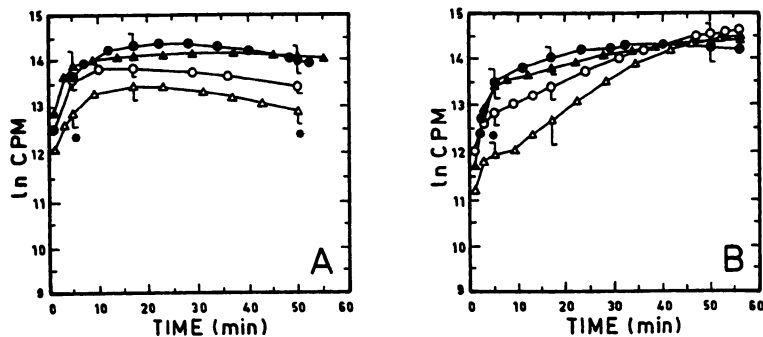


Fig. 7. Lucigenin-enhanced chemiluminescence of PMN from B10.A (Δ , \blacktriangle) and A/J (\circ , \bullet), obtained 24 h (A) and 15 days (B) after air pouch infection (PMA, filled symbols; Pb, open symbols). *Statistical differences between mouse strains (A/J and B10.A).

PMN from B10.A mice. It is interesting to note that 15 days after air pouch infection with *P. brasiliensis*, an expressive number of viable yeasts was recovered only from the susceptible mice.

DISCUSSION

The H_2O_2 -MPO-halide system from PMN cells and from cell-free medium is an effective killing mechanism for opportunistic [18, 19] and dimorphic fungi [7, 12–16]. Air pouch inoculation in animal models provides an additional useful tool to study the interaction of PMN and *P. brasiliensis* yeast cells, since by this route a cellular population rich in neutrophils could be obtained, similar to what occurs after inoculation of lipopolysaccharide [28].

In the present work, we studied the oxidative burst of PMN present in the air pouch of B10.A and A/J mice, as well as the ability of this cell population to inhibit *P. brasiliensis* growth. Two types of stimuli able to trigger O_2^- release by neutrophils were used: PMA and Pb. The luminogenic probes, luminol and lucigenin, are commonly used to monitor PMN activation by detecting, respectively, the H_2O_2 -MPO system and O_2^- [20–22, 33].

The results herein obtained show that, during infection, the PMN present in the air pouch of A/J mice become progressively more activated when stimulated in vitro with killed *P. brasiliensis* yeast cells. This stimulation can easily be observed by the increase of the luminol-enhanced CL of cells obtained 15 days after infection when compared with those recovered after 24 h. This activation in the course of infection was not observed with the B10.A mice, constituting the main difference in respect to PMN behavior between the B10.A and A/J mice.

The effects observed by the addition of catalase upon the luminol-enhanced CL clearly showed that the production of ROS depends on the stimulus utilized. When the soluble stimulus PMA was used, at any time of infection, the effect of catalase clearly showed an increasing H_2O_2 production along with the oxidative burst. However, when Pb was used as stimulus, PMN isolated 24 h after the infection were not able to release an expressive amount of H_2O_2 in the medium, since catalase had no effect in this experimental group. After 15 days of infection, only the PMN of A/J mice were able to show an early response with release of large amounts of H_2O_2 .

In vitro stimulation of PMN from A/J mice with Pb showed that this cell population produces more O_2^- than that obtained from B10.A mice. In this strain the H_2O_2 -MPO-halide system is inefficient, as evidenced by depressed luminol-enhanced CL. It is noteworthy that a decrease in the spontaneous lucigenin CL after 15 days of infection occurred only with B10.A cells. This indicates that, during the infection, the PMN from this mouse strain lose their ability to produce O_2^- . The differences in the formation of reactive oxygen species were evidenced only when PMN from B10.A were activated in vitro with Pb and not when PMA was employed. A more pronounced activation of PMN from A/J mice than of PMN from B10.A mice was also obtained when zymozan particles were employed for the in vitro activation of PMN obtained from mice 15 days after s.c. infection (data not shown). Moreover, no differences in the phagocytic activity of these cells could be detected; both PMN obtained from resistant and susceptible mice were able to engulf opsonized zymozan particles (data not shown).

Macrophages are noted for their paucity in MPO (reviewed in refs. 33–35) and for their inefficiency to kill *P. brasiliensis* yeasts, allowing their intracellular multiplication [36]. However, when macrophages are activated in vitro by interferon- γ these cells are able to kill *P. brasili-*

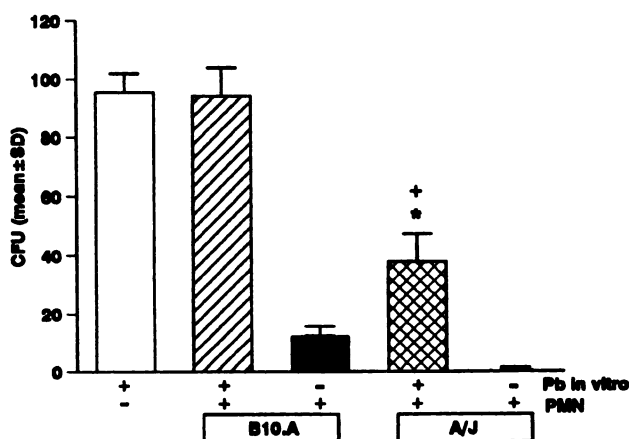


Fig. 8. Effect of co-cultivation of PMN from s.c. air pouch of B10.A and A/J mice with yeast cells of the virulent Pb18 *P. brasiliensis* isolates in a 50:1 proportion, expressed as mean \pm SE of CFU counts. Controls consisted of similarly obtained PMN, without exposition to *P. brasiliensis* and of *P. brasiliensis* suspensions. *Statistical difference between the mouse strains (A/J and B10.A); + statistical difference in relation to *P. brasiliensis* suspension.

iensis by a mechanism independent of ROS production [37]. Our precaution in eliminating the adherent cell population in the killing experiments allows us to attribute the fungicidal role to PMN. Although 10–20% of the cells in the exudate are mononuclear, our data clearly show that PMN from resistant mice are the most important source of ROS in the air pouch lesion of A/J mice.

In parallel with these alterations, the susceptible mice were unable to markedly inhibit viable *P. brasiliensis* yeast growth, after in vitro co-cultivation with their PMN. This data, which shows a fundamental role of PMN in the control of paracoccidioidomycosis, corroborate previously obtained ones, employing athymic and euthymic mice of susceptible BALB/c background, which showed that during the first week of infection, when PMNs were abundant in the lesions, *P. brasiliensis* dissemination was under control and there was presence of fungal debris near these phagocytic cells [23].

The participation of PMN in resistance to *P. brasiliensis* infection may well extend to further stages of the evolution of the disease. In fact, the role of PMN in the immune phase of the response against some infectious agents has recently been demonstrated, as it was shown that BALB/c mice were unable to express T cell-mediated immunity to *Listeria monocytogenes* in the absence of granulocytes [38]. Moreover, a recent study [39] described that stimulated human PMN produced interleukin-12, which may play an important role at the site of infection, inducing T and natural killer cells to produce interferon- γ , the classical macrophage activator.

In our system, the PMN present in the air pouch can be subject to the effect of various cytokines produced by different cells such as the keratinocytes, mast cells, T lymphocytes, macrophages, natural killer, and PMN (reviewed in refs. 37–44). These cells can produce cytokines such as interleukin-1, interleukin-3, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , and interferon- γ , which activate the oxidative burst and the fungicidal activity of PMN [18, 19, 44]. It is possible that the differences appointed in the present study reflect the qualitative or quantitative differences in the stimulatory cytokines present in the air pouches of the two mouse strains studied.

In contrast to the i.p. route, the s.c. route of *P. brasiliensis* inoculation leads to adequate delayed-type hypersensitivity responses and survival of both A/J and B10.A mice [2]. At 15 days of air pouch infection, both mouse strains showed intense delayed-type hypersensitivity (data not shown). Therefore, the PMN of these mice can be subject to the effects of various cytokines, which can activate or down-regulate the oxidative burst and the fungicidal activity of these cells.

PMN are now accepted as participating not only in the initial, natural immune response, but also in the subsequent immune phase of anti-infectious resistance. Seen under this light, our present results suggest that PMN of the resistant mice, being more activated and also able to better control *P. brasiliensis* growth than those of the sus-

ceptible ones, can, at the initial stages of infection, effectively diminish the fungal load. During later stages, the PMN of the resistant mice may be more effective in promoting and/or participating in cell interactions that result, ultimately, in the establishment of protective immunity. In conclusion, the high amount of activated PMN in vivo in air pouch and ROS production by PMN from A/J cells points to a central role of these cells in the fast resolution of *P. brasiliensis* infection. Moreover, the H₂O₂-MPO-halide system appears to be an important source of oxidant species contributing to *P. brasiliensis* killing and confirm earlier data that shows that deficiency in MPO leads to increased susceptibility to fungal infections.

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REFERENCES

1. Restrepo, A. (1988) Immune response to *Paracoccidioides brasiliensis* in human and animal hosts. In *Current Topics in Medical Mycology*. (M.R. McGinnis, ed.), vol. 2, Springer-Verlag, New York, 239–275.
2. Calich, V.L.G., Singer-Vermes, L.M., Russo, M., Vaz, C.A.C., Burger, E. (1993) Immunogenetics in paracoccidioidomycosis. In: *Paracoccidioidomycosis*. (M.F. Franco, C.S. Lacaz, A. Restrepo-Moreno, G. Del Negro, eds.) CRC Press, Miami, 151–168.
3. Calich, V.L.G., Kipnis, T.L., Mariano, M., Fava Neto, C., Dias da Silva, W. (1979) The activation of the complement system by *Paracoccidioides brasiliensis* in vitro: its opsonic effect and possible significance for an in vivo model of infection. *Clin. Immun. Immunopathol.* 12, 20–30.
4. Calich, V.L.G., Vaz, C.A.C., Burger, E. (1985) PMN chemotactic factor produced by glass adherent cells in the acute inflammation caused by *Paracoccidioides brasiliensis*. *Br. J. Exp. Pathol.* 66, 57–65.
5. Brummer, E., Castaneda, E., Restrepo, A. (1993) Paracoccidioidomycosis: an update. *Clin. Microb. Rev.* 6, 39–117.
6. Restrepo, A., Velez, H. (1975) Efectos de la fagocitosis in vitro sobre *Paracoccidioides brasiliensis*. *Sabouraudia* 13, 10–21.
7. McEwen, J.G., Brummer, E., Stevens, D.A., Restrepo, A. (1987) Effect of murine polymorphonuclear leukocytes on the yeast form of *P. brasiliensis*. *Am. J. Trop. Med. Hyg.* 36, 603–608.
8. Brummer, E., McEwen, F.G., Stevens, D.A. (1986) Fungicidal activity of murine inflammatory polymorphonuclear neutrophils, comparison with murine peripheral blood PMN. *Clin. Exp. Immunol.* 66, 681–690.
9. Brummer, E., Sugar, A.M., Stevens, D.A. (1984) Immunological activation of polymorphonuclear neutrophils for fungal killing: studies with murine cells and *Blastomyces dermatitidis* in vitro. *J. Leukoc. Biol.* 36, 505–520.
10. Gohman-Yahr, M., Essensfeld-Yahr, E., Alborno, M.C. (1980) Defect of in vitro digestive ability of polymorphonuclear leukocytes in paracoccidioidomycosis. *Infect. Immun.* 28, 557–566.
11. Schaffner, A., Davis, C.E., Schaffner, T., Markert, M. (1986) In vitro susceptibility of fungi to killing by neutrophil granulocytes discriminates between primary pathogenicity and opportunism. *J. Clin. Invest.* 78, 511–524.
12. McEwen, J.G., Sugar, A.M., Brummer, E., Restrepo, A., Stevens, D.A. (1984) Toxic effect of products of oxidative metabolism on the yeast form of *P. brasiliensis*. *J. Med. Microb.* 18, 423–428.
13. Sugar, A.M., Chahal, R.S., Brummer, E., Stevens, D.A. (1983) Susceptibility of *Blastomyces dermatitidis* strains to products of oxidative metabolism. *Infect. Immun.* 41, 908–912.
14. Brummer, E., Sugar, A.M., Stevens, D.A. (1985) Enhanced oxidative burst in immunologically activated but not elicited polymorphonuclear leukocytes correlates with fungicidal activity. *Infect. Immun.* 49, 396–441.
15. Brummer, E., Kurita, N., Yoshida, S., Nishimura, K., Miyaji, M. (1992) A basis for resistance of *Blastomyces dermatitidis* killing by human neutrophils: inefficient generation of myeloperoxidase system products. *J. Med. Vet. Mycol.* 30, 233–243.

16. Kurita, N., Terao, K., Brummer, E., Ito, E., Nishimura, K., Miyaji, M. (1991) Resistance of *Histoplasma capsulatum* to killing by human neutrophils, evasion of oxidative burst and lysosome fusion products. *Mycopathologia* 115, 207–213.
17. Wagner, D.K., Collins-Lech, C., Sohnle, P.G. (1986) Inhibition of neutrophil killing of *Candida albicans* pseudohyphae by substances which quench hypochlorous acid and chloramines. *Infect. Immun.* 51, 731–735.
18. Diamond, R.D., Lyman, C.A., Wysong, D.R. (1991) Disparate effects of interferon-gamma and tumor necrosis factor-alpha on early neutrophil respiratory burst and fungicidal responses to *Candida albicans* hyphae in vitro. *J. Clin. Invest.* 87, 711–720.
19. Kowanko, I.C., Ferrante, A., Harvey, D.P., Carman, K.L. (1991) Granulocyte-macrophage colony-stimulating factor augments neutrophil killing of *Torulopsis glabrata* and stimulates neutrophil respiratory burst and degranulation. *Clin. Exp. Immun.* 83, 225–230.
20. Allen, R.C., Loose, L.D. (1976) Phagocytic activation of luminol dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. *Biochem. Biophys. Res. Commun.* 69, 245–252.
21. Faulkner, K., Fridovich, I. (1993) Luminol and lucigenin as detectors for O₂⁻. *Free Rad. Biol. Med.* 15, 447–451.
22. Mikenberg, I., Ferber, E. (1984) Lucigenin dependent chemiluminescence as a new assay for NADPH oxidase activity in particulate fractions of human polymorphonuclear leukocytes. *J. Immun. Meth.* 71, 61–67.
23. Lenzi, H.L., Calich, V.L.G., Miyaji, M., Sano, A., Nishimura, K., Burger, E. (1994) Fibrosis patterns of lesions developed by athymic and euthymic mice infected with *Paracoccidioides brasiliensis*. *Braz. J. Med. Biol. Res.* 27, 2301–2306.
24. Kashino, S.S., Singer-Vermees, L.M., Calich, V.L.G., Burger, E. (1990) Alterations in the pathogenicity of one *Paracoccidioides brasiliensis* isolate do not correlate with its in vitro growth. *Mycopathologia*, 111, 173–180.
25. Fava Netto, C. (1961) Contribuição para o estudo imunológico da blastomicose de Lutz. *Rev. Inst. Adolfo Lutz* 21, 99–194.
26. Kashino, S.S., Calich, V.L.G., Singer-Vermees, L.M., Abrahamsohn, P.A., Burger, E. (1987) Growth curves, morphology and ultrastructure of ten *Paracoccidioides brasiliensis* isolates. *Mycopathologia* 99, 119–128.
27. Berliner, M.D., Reza, M.E. (1966) Vital staining of *Histoplasma capsulatum* with Janus Green B. *Sabouraudia* 5, 26–29.
28. Harmsen, A.G., Havell, E.A. (1990) Roles of tumor necrosis factor and macrophages in lipopolysaccharide-induced accumulation of neutrophils in cutaneous air pouches. *Infect. Immun.* 52, 297–302.
29. Carson, F.L., Martin, J.H., Lynn, J.A. (1973) Formalin fixation for electron microscopy. A re-evaluation. *Am. J. Clin. Pathol.* 59, 365–373.
30. Castañeda, E., Brummer, E., Perlman, A.M., McEwen, J.C., Stevens, D.A. (1988) A culture medium for *Paracoccidioides brasiliensis* with high plating efficiency, and the effect of siderophores. *J. Med. Vet. Mycol.* 26, 351–358.
31. Singer-Vermees, L.M., Ciavaglia, M.C., Kashino, S.S., Burger, E., Calich, V.L.G. (1992) The source of the growth-promoting factor(s) affects the plating efficiency of *P. brasiliensis*. *J. Med. Vet. Mycol.* 30, 261–264.
32. Zar, J.H. (1984) *Biostatistical Analysis*, 2nd ed., Prentice-Hall, NJ.
33. Brestel, E.P. (1987) Mechanisms of cellular chemiluminescence. In *Cellular Chemiluminescence*, vol. 1, chapt. 9, 93–103.
34. Peterson, P.K., Gaziano, E., Suh, H.J., Devalon, M., Petersen, L., Krane, W.F. (1985) Antimicrobial activities of dialysate-elicited and resident human peritoneal macrophages. *Infect. Immun.* 49, 212–218.
35. Rosen, G.M., Pou, S., Ramos, C.L., Cohen, M.S., Britigan, B.E. (1995) Free radicals and phagocytic cells. *FASEB J.* 9, 200–209.
36. Brummer, E., Hanson, L.H., Restrepo, A., Stevens, D.A. (1989) Intracellular multiplication of *P. brasiliensis* in macrophage killing and restriction of multiplication by activated macrophages. *Infect. Immun.* 57, 2289–2294.
37. Brummer, E., Hanson, L.H., Stevens, D.A. (1988) Gamma interferon activation of macrophages for killing of *Paracoccidioides brasiliensis* and evidence for nonoxidative mechanisms. *Int. J. Immunopharmacol.* 10, 945–952.
38. Appelberg, R., Castro, A.G., Silva, M.T. (1994) Neutrophils as effector cells of T-cell-mediated, acquired immunity in murine listeriosis. *Immunology* 83, 302–307.
39. Cassatella, M.A., Meda, L., Casperini, S., D'Andrea, A., Ma, X., Trinchieri, G. (1995) Interleukin-12 production by human polymorphonuclear leukocytes. *Eur. J. Immun.* 25, 1–5.
40. Bos, J.D., Kapsenberg, M.L. (1993) The skin immune system: progress in cutaneous biology. *Immun. Today* 14, 75–78.
41. Ribeiro, R.A., Flores, L.A., Cunha, F.Q., Ferreira, S.H. (1991) IL-8 causes in vivo neutrophil migration by a cell dependent mechanism. *Immunology* 73, 472–477.
42. Djeu, J.Y. (1992) Cytokines and anti-fungal immunity. *Adv. Exp. Med. Biol.* 319, 217–223.
43. Lloyd, A.R., Oppenheim, J.J. (1992) Poly's lament: the neglected role of the polymorphonuclear neutrophil in the afferent limb of the immune response. *Immun. Today* 13, 169–172.
44. Sullivan, C.W., Carper, H.T., Mandell, G.L. (1993) The effect of three human recombinant hematopoietic growth factors (granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor and interleukin-3) on phagocyte oxidative activity. *Blood* 81, 1863–1970.