Importance of complement 3 and mannose receptors in phagocytosis of *Paracoccidioides brasiliensis* conidia by *Nramp1* congenic macrophages lines

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Abstract

Genetic factors influence susceptibility to Paracoccidioidomycosis, a Latin American endemic mycosis. The pattern of susceptibility of congenic mouse strains infected with *Paracoccidioides brasiliensis* resembles the pattern of the *Nramp1* gene. Thus, congenic murine bone-marrow-derived macrophage lines B10R (*Nramp1*^{rGly169}) and B10S (null Nramp1 protein expression, *Nramp1*^{sAsp169}) were infected with *P. brasiliensis* conidia and compared, under opsonic and nonopsonic conditions. Opsonization increased the percentage of phagocytosis by both cell lines. B10R macrophages exhibited a higher percentage of cells with associated conidia and higher number of conidia per macrophage than B10S. Heat-inactivation and EDTA treatment of serum used for opsonization, and treatment of macrophages with anti-complement receptor 3 (CR3) decreased phagocytosis by both cell lines. α-methyl-D-mannoside reduced phagocytosis by B10R macrophages, suggesting that the mannose receptor participates in phagocytosis by these cells. The CR3 expression was similar on both cell lines and B10R expressed more mannose receptors, but neither cell line expressed CR1. IFNγ decreased the conversion of conidia to the yeast form of *P. brasiliensis* in B10R, but not in B10S macrophages.

Introduction

Paracoccidioidomycosis (PCM), a severe endemic mycosis frequently diagnosed in Latin America (Restrepo & Tobón, 2005), is caused by the dimorphic fungus *Paracoccidioides brasiliensis*, a facultative intracellular microorganism. Epidemiological and experimental evidence suggests that natural infection is initiated after inhalation of the conidia produced by the mycelial form of the fungus (Restrepo & Tobón, 2005). In the lung, conidia phagocytosed by alveolar macrophages begin to transform into yeast cells after 12–18 h (McEwen *et al.*, 1987). *In vitro*, conidia and yeasts of *P. brasiliensis* are efficiently phagocytosed by murine (Cano *et al.*, 1992) and human (Moscardi-Bachi *et al.*, 1993) macrophages. Once ingested by nonactivated phagocytes, the fungus transforms and replicates intracelullarly; but when these cells are activated with cytokines the transition of conidia to yeast and the budding of yeasts are inhibited (Cano *et al.*, 1992; Moscardi-Bachi *et al.*, 1993). However, it has been shown that some fungal compounds, such as gp70 (de Mattos Grosso *et al.*, 2003) and melanin (da Silva *et al.*, 2005) are able to inhibit yeast uptake by alveolar and peritoneal macrophages, demonstrating the complex interactions between *P. brasiliensis* and the host cells.

Genetically controlled factors are known to regulate the course and progression of infection with *P. brasiliensis* (Calich *et al.*, 1994). A comparison of different congenic strains of mice, infected intraperitoneally with *P. brasiliensis* yeast cells, showed a differential pattern of resistance and susceptibility, as determined by survival time. The A/Sn strain of mice was shown to be the most resistant to infection with *P. brasiliensis*, while B10D2/nSn, B10.A and B10D2/oSn strains were much more susceptible than A/Sn. Susceptibility was not dependent on the size of inocula, and it was not X-linked (Calich *et al.*, 1985). Susceptibility was

not controlled by the major histocompatibility complex (MHC), H-2, as the most resistant (A/Sn) and the most susceptible (B10.A) strains had the same H-2^a genotype. Interestingly, the distribution pattern of resistance/susceptibility (R/S) to PCM among congenic mouse strains (Calich *et al.*, 1987) resembled the pattern observed with the *Bcg/Nramp1* gene (recently renamed Slc11a: solute carrier family 11) (Wyllie *et al.*, 2002) after infection with the intracellular microorganisms *Salmonella typhimurium*, *Mycobacterium bovis* and *Leishmania donovani* (O'Brien *et al.*, 1980; Skamene *et al.*, 1982). Phenotypically, macrophages from resistant A/Sn mice showed a higher microbicidal activity than macrophages from susceptible B10.A mice that do not express the Nramp1 protein (Vidal *et al.*, 1996a, b), further supporting the hypothesis that the *Nramp1* gene might be one of the putative genes controlling R/S to *P. brasiliensis* infection.

It has been shown that the *Nramp1* gene controls the production of reactive nitrogen intermediates (RNI). The bone-marrow-derived macrophage line (B10R) from B10.Bcg^r resistant mice expressing resistant alleles of the *Nramp1* gene ($Nramp1^{Gly169}$) produced a higher amount of nitric oxide (NO) than the B10S macrophage cell line derived from mice that do not express the Nramp1 protein (B10A.$Bcg^{s\ Asp169}$) following the same stimulation with IFN-γ or infection with *Mycobacterium bovis* BCG (Barrera *et al.*, 1994) or *Mycobacterium tuberculosis* H37Rv (Arias *et al.*, 1997). In murine macrophages, NO production is induced during *P. brasiliensis* infection (Bocca *et al.*, 1998; Nascimento *et al.*, 2002). Furthermore, IFN-γ activated murine macrophages kill *P. brasiliensis* conidia by L-arginine–NO dependent mechanisms (González *et al.*, 2000).

These observations prompted us to determine whether these two types of macrophage lines exhibit different responses after infection with *P. brasiliensis* conidia and also if there would be a possible mechanism responsible for the different ability of these two macrophage lines to control infection with *P. brasiliensis*. The results presented in this manuscript demonstrate important differences in the phagocytic potential between macrophages that express the Nramp1 protein (B10R macrophages) and macrophages that do not express the Nramp1 protein (B10S macrophages). A differential effect of IFN-γ on the ability of B10R and B10S macrophages to control *P. brasiliensis* conidia to yeast form conversion is also documented in our manuscript.

Materials and methods

Reagents

Dulbecco's modified Eagle's Medium (DMEM) and L-glutamine were purchased from GIBCO-BRL (Life Technologies, Grand Island, NY), fetal bovine serum (FBS) from Biowhittaker, (Walkersville, MD), Percoll from Amersham, Pharmacia Biotech AB (Uppsala, Sweden), normal murine serum (NMS) and recombinant murine Interferon-γ (IFN-γ) from Fitzgerald Industries International, Inc. (Concord, MA). Penicillin–streptomycin was purchased from Irvine Scientific (Santa Ana, CA); Fc receptor blocking antibody (CD32/16 – Fcγ II/III receptor; clone CT-17.1, CT-17.2) and goat anti-rat IgG labeled with fluorescein isothiocyanate (FITC) from CALTAG (Burlingame, CA); rat anti-mouse CD11b (anti-CR3; clone M1/70 Ig $G_{2b,\kappa}$), rat anti-mouse CD35 (anti-CR1; clone 8C12 Ig $G_{2a,\kappa}$) and rat $IgG_{2a,\kappa}$ isotype control (clone R35-95) from BD-Pharmingen (San Diego, CA). Mannosylated albumin labeled with phycoerytrin (PE), biotin-labeled albumin, PE–streptavidin and α-methyl-D-mannoside were purchased from Sigma (St Louis, MO). Sabouraud/Dextrose Modified (Mycosel Agar) and Bacto-Agar were purchased from BBL (Beckton Dickinson, Sparks, MD). Media, sera, and reagents used in macrophage cultures were assayed for the presence of endotoxin by the quantitative *Limulus amebocyte* Lysate (LAL) assay using the QCL-1000 kit purchased from BioWhitaker (Walkersville, MD).

Culture and maintenance of murine macrophage lines

B10S and B10R macrophage lines were generated from the bone marrow of B10A.$Nramp1^{sAsp169}$ mice that do not express the Nramp1 protein (10), and B10A.$Nramp1^{rGly169}$ mice, respectively, as previously described (Radzioch *et al.*, 1991). The cell cultures were maintained in DMEM, supplemented with 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 μM L-glutamine, at 37 °C and 5% CO_2. The medium was changed three times per week and the cells were maintained at a concentration of less than 1×10^6 cells mL^{-1}. Prior to each experiment, the cells were collected when the cell concentration reached $0.8–1.0 \times 10^6$ mL^{-1}, by scraping with a rubber policeman (Sarstedt, Newton, NC) and the cell viability was determined by trypan blue exclusion and was always greater than 95%. The cells were maintained up to 15 generations in culture and subsequently, cultures were re-started from frozen vials. The responsiveness of each newly restarted frozen vial culture was monitored by testing the nitric oxide production in supernatants harvested from cell cultures stimulated with IFN-γ (100 U mL^{-1}) for 24 h, as previously described (Ding *et al.*, 1988).

Culture and production of *Paracoccidioides brasiliensis* conidia

Mycelial cultures of *P. brasiliensis* (isolate Gr, ATCC, No. 60885) obtained from a Colombian patient with paracoccidioidomycosis were used for this study (Restrepo *et al.*, 1986). The fungus was maintained in its mycelial phase at

18 °C in solid Sabouraud/Dextrose Modified Agar (Beckton Dickinson, Sparks, MD) and changed to fresh medium every two weeks; the Synthetic McVeigh-Morton (SMV) medium (Restrepo & Jiménez, 1980) was used to increase the fungus replication capacity. The mycelial cultures in solid SMV medium were passed to SMV liquid medium. These cultures were incubated at 18 °C on a reciprocating shaker at 150 r.p.m. for 2 weeks. After this time, they were homogenized, and the resulting suspension plated on Petri dishes containing culture media poor in carbohydrates, such as water-agar and glucose/salts agar (Bustamante-Simon *et al.*, 1985). The cultures were then incubated at 18 ± 4 °C for 2 months. After this time, plates that were free of contamination and exhibited conidia were used. To obtain the conidia, 10 mL of sterile PBS containing 0.15 M NaCl, 0.01% Tween 20, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin were added to flood these cultures. This procedure was repeated three times for 30 mL of final volume by plate. The suspension was placed in sterile screw-capped Erlenmeyer flasks containing 3 mm glass beads and then shaken in a reciprocating shaker at 250 r.p.m. for 45 min at room temperature. Then, the suspension was centrifuged at 1500 ***g*** at 4 °C for 30 min. To optimize the detachment of the conidia from mycelial fragments, the pellet was diluted in 10 mL of PBS and sonicated twice at 7 Hz for 15 s at 4 °C (model 200, Branson Ultrasonic Co., Danbury, CT).

Isolation of *Paracoccidioides brasiliensis* conidia by Percoll gradients

Conidia were recovered as previously described (Jiménez Mdel *et al.*, 2004). Briefly, after sonication, the pellet corresponding to each plate was diluted in 35 mL of PBS, layered over 10 mL of 95% Percoll in 0.25 M sucrose and centrifuged at 1500 ***g***, 4 °C for 60 min. Conidia was recovered in the pellet, but the interphase Percoll-PBS was also recovered, diluted in PBS and centrifuged on a new Percoll gradient. The two pellets were pooled and washed twice with PBS at 1500 ***g***, 4 °C for 30 min. The conidia were counted and the viability determined with fluorescein–ethidium bromide (Calich *et al.*, 1978). The purity and viability were always ≥90%. Conidia were opsonized with 30% normal murine serum (NMS) for 30 min at 37 °C (Calich *et al.*, 1979). After incubation, the conidia were diluted in DMEM to obtain the required number of conidia for every ratio of infection.

Phagocytosis of *Paracoccidioides brasiliensis* conidia by B10R and B10S macrophages

Cell cultures were adjusted to 1.2×10^5 macrophages per well in eight-well Lab-Tek slides (Nunc, Inc. Naperville, IL). Macrophages were infected with nonopsonized or opsonized conidia of *P. brasiliensis* at ratios of conidia to macrophage 1 : 1, 2 : 1 and 5 : 1 and incubated for 6 and 12 h at 37 °C and 5% CO_2. At the corresponding times, the slides were washed with DMEM at 37 °C, fixed with absolute methanol, stained with silver metenamine (Gomori) and contrasted with 0.1% Toluidine blue. The percentage of macrophages with *P. brasiliensis* conidia and the number of conidia per infected macrophage were determined by counting 500 macrophages at 1000× magnification.

Identification of opsonins involved in phagocytosis of *Paracoccidioides brasiliensis* conidia by B10R and B10S macrophages

To determine the nature of the opsonins facilitating phagocytosis, *P. brasiliensis* conidia were incubated for 20 min at 37 °C with NMS, NMS mixed with 20 mM EDTA (Calich *et al.*, 1979; Zabaleta *et al.*, 1998) or NMS inactivated at 56 °C for 30 min. Thereafter, these conidia were used to infect macrophages at 2 : 1 macrophage : conidia ratio as described below. To determine the role of the CR1 (CD35) and CR3 (CD11b, CD18) receptors, macrophages were plated in eight-well Lab-Tek slides (Nunc, Inc. Naperville, IL) at 1.2×10^5 macrophages per well for 2 h. Thereafter, anti-CR1 (15 μg mL^{-1}), anti-CR3 (10 μg mL^{-1}), anti-CR1 plus anti-CR3 and isotype control (10 μg mL^{-1}) were added for 30 min at 4 °C. The cells were infected with opsonized conidia (conidia : macrophage 2 : 1) for 6 h. The slides were stained with Gomori's silver and counter-stained with 0.1% Toluidine blue as described above and the percentage of macrophages with associated conidia and the fungal burden per infected cell was determined by light microscopy as described above.

Effect of α-methyl-D-mannoside (α-MM) on phagocytosis of *Paracoccidioides brasiliensis* conidia by B10R and B10S macrophages

B10R and B10S macrophages were plated on eight-well Lab-tek slides at 1.2×10^5 cells per well in 0.1 mL for 2 h at 37 °C in 5% CO_2. Then, different doses of α-MM (2.5, 5 and 10 μg mL^{-1}) (Zabaleta *et al.*, 1998) were added and the cells cultured for 1 h. Thereafter, macrophages were infected with opsonized *P. brasiliensis* conidia (2 : 1) for 6 h at 37 °C in 5% CO_2. The percentage of macrophages with associated *P. brasiliensis* and the fungal burden per infected cell were determined as described above.

Determination of CR1, CR3 and mannose receptor expression by flow cytometry

Three hundred thousand cells were incubated with 1 μg mL^{-1} of anti-Fcγ receptor (CD32/16) for 15 min at 4 °C.

Then, the cells were washed twice with PBS that contained 1% sodium azide and 1% bovine serum. Thereafter, the cells were incubated with either 1 μg of anti-CR1, 1 μg of anti-CR3 or 5 μg of $IgG_{2a,\kappa}$ isotype control for 30 min at 4 °C followed by incubation with goat anti-mouse IgG-FITC for 30 min at 4 °C. To determine the expression of the mannose receptor, the macrophages were incubated for 20 min with 20 μg mL^{-1} PE-labeled mannosylated albumin. As control, the cells were incubated with biotin-labeled albumin, followed by PE-streptavidin. The cells were washed and their fluorescence determined in a Coulter Epics XL flow cytometer (Coulter-Beckman, Miami, FL). The macrophages were acquired by size (FCS) and granularity (SSC), FL1 (FITC) and FL2 (PE), respectively. 10 000 events were counted by sample. The results are presented as the percentage of positive cells and the mean of fluorescence intensity (MFI).

Conversion of *Paracoccidioides brasiliensis* conidia

Macrophages were infected with opsonized and nonopsonized conidia at 2 : 1 conidia : macrophage ratio for 96 and 120 h with or without 50 or 100 U mL^{-1} of recombinant murine IFN-γ. After incubation, the supernatants were removed and kept at −70 °C to determine nitrite concentration. The macrophages were fixed and stained as described above. To determine the conversion, ≥300 fungal cells associated with macrophages were counted and the fungus morphology recorded (conidia, transitional form, yeast or multiple budding yeasts cells). The percentage of converted cells was calculated in relation to the total fungal cells by smear.

Statistical analysis

Comparisons between the different treatments were done by one- and two-way analysis of variance. The Tukey's test and unpaired *t*-test were used for comparison between groups. For all analysis the software Prism 3 (GraphPad Software, San Diego, CA) was used.

Results

Determination of the capacity of murine macrophage lines B10R and B10S to phagocytose *Paracoccidioides brasiliensis* conidia

To determine whether B10R and B10S macrophages differ in their capacity to phagocytose *Paracoccidioides brasiliensis* conidia, macrophages were infected with *P. brasiliensis* conidia, either opsonized or nonopsonized, and cocultured for 6 and 12 h, at different conidia–macrophage ratios (1 : 1, 2 : 1 and 5 : 1). For both cell lines, phagocytosis was always 17 to 20% higher for opsonized than for nonopsonized propagules, irrespective of time and infectious dose (Fig. 1a). B10R macrophages exhibited a higher percentage of macrophages with associated conidia than B10S cells derived from mice that do not express the *Nramp1* protein, under opsonized ($P < 0.0001$) and nonopsonized ($P < 0.009$) conditions (Fig. 1a). At the highest infectious ratio studied (5 : 1) B10R showed 82.2 ± 3.8% and 89.5 ± 2.2% of phagocytosis, at 6

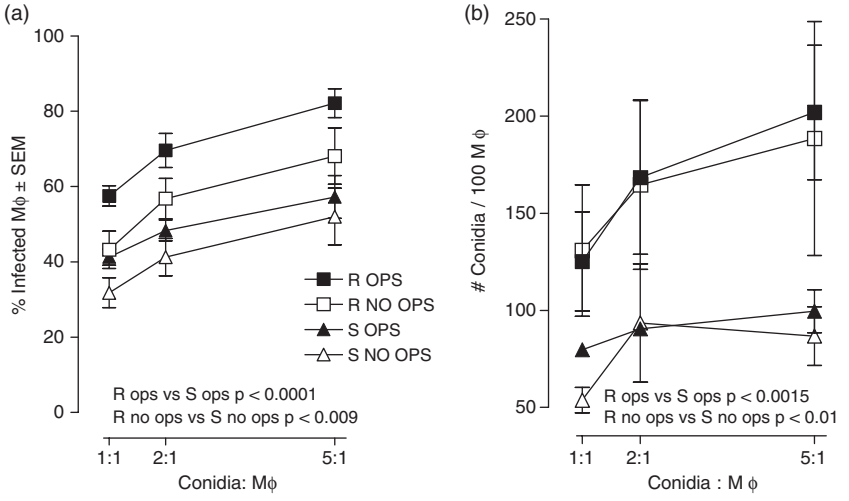

Fig. 1. Phagocytosis of opsonized and nonopsonized *Paracoccidioides brasiliensis* conidia by B10R and B10S macrophages. Macrophages (B10R, squares; B10S, triangles) were infected for 6 h with *P. brasiliensis* conidia opsonized (closed symbols) or nonopsonized (open symbols) at different conidia: macrophage ratios. The slides were stained with silver metenamine and contrasted with toluidine blue. The percentage of macrophages with associated *P. brasiliensis* conidia (a) and the number of conidia per 100 infected macrophages were determined (b) ($n = 5$). The results were analyzed by two-way ANOVA.

Table 1. Distribution of the percentage of B10R and B10S macrophages phagocytosing different number of conidia

	B10R				B10S			
MOI	0*	1–2	3–4	≥5	0	1–2	3–4	≥5
Opsonized								
1 : 1	47.5 ± 10.3†	36.6 ± 4.8	11.3 ± 3.6	4.6 ± 2.5	56.9 ± 1.4	36.9 ± 2.2	5.1 ± 0.9	1.0 ± 0.2
2 : 1	37.4 ± 9.4	39.8 ± 2.5	11.2 ± 2.6	11.6 ± 4.5	51.0 ± 2.8	41.0 ± 2.0	7.0 ± 1.0	1.0 ± 0.2
5 : 1	26.8 ± 7.5	40.7 ± 3.0	17.8 ± 2.0	14.7 ± 6.4	52.0 ± 7.0	39.3 ± 3.7	7.6 ± 3.0	1.1 ± 0.3
Nonopsonized								
1 : 1	52.9 ± 5.4	35.3 ± 1.9	9.1 ± 2.9	2.7 ± 1.7	69.2 ± 3.8	29.2 ± 3.1	1.5 ± 0.6	0.1 ± 0.1
2 : 1	40.3 ± 9.4	34.6 ± 4.0	14.2 ± 5.3	10.6 ± 4.5	57.7 ± 3.7	38.2 ± 3.3	3.4 ± 1.8	0.7 ± 0.4
5 : 1	47.1 ± 7.5	35.3 ± 2.2	9.3 ± 2.2	8.3 ± 4.7	65.6 ± 0.7	29.6 ± 0.5	4.0 ± 0.4	0.8 ± 0.1

*Number of conidia per cell.
†Percentage of cells ± SEM.
MOI, multiplicity of infection.

and 12 h respectively, compared to 57.3 ± 5.6 % and 63.5 ± 2.2% for B10S macrophages.

To further examine the differences in phagocytosis between the two cell lines, the number of conidia per macrophage was counted (Fig. 1b, Table 1). B10R cells showed higher number of conidia per macrophage than B10S both under opsonized ($P < 0.0015$) and nonopsonized conditions ($P < 0.01$) (Fig. 1b). The number of conidia phagocytosed by B10R macrophages increased with the size of inoculum, whereas such a correlation was not found with B10S macrophages. However, opsonization did not significantly affect the number of conidia per macrophage in either cell line. The results of the fungal burden analysis shown in Table 1 demonstrate that there was a higher percentage of B10R cells phagocytosing more than three conidia than B10S cells. These results suggest that the phagocytic capacity, as measured by the percentage of infected cells and the load of conidia per phagocyte, is higher for macrophages expressing the Nramp1 protein (B10R) than for macrophages derived from mice that do not express the Nramp1 protein (B10S)

Effect of complement inactivation and CR1 and CR3 blockade on phagocytosis of *Paracoccidioides brasiliensis* conidia

It has been reported that phagocytosis of *P. brasiliensis* yeast forms is complement-dependent (Calich *et al.*, 1979). To identify the type of opsonin involved in ingestion and/or adherence of conidia, B10R and B10S macrophages were infected with conidia opsonized with heat-inactivated mouse serum or treated with EDTA, comparatively with nontreated mouse serum and nonopsonized conidia (Fig. 2). For both the cell lines, serum heat-inactivation and EDTA treatment diminished ($P < 0.05$) the percentage of phagocytosis to the level observed without opsonization ($P < 0.01$) (Fig. 2a). These treatments also resulted in a reduced number of intracellular conidia (B10R, $P < 0.001$; B10S, $P < 0.05$) (Fig. 2b).

These results suggest that opsonization of *P. brasiliensis* conidia also is complement-dependent. Thus, to determine the complement receptors involved in this process, the blocking of complement receptors 1 and 3 using specific monoclonal antibodies was attempted (Fig. 2c and d). In both cell lines, isotypic control had no effect. However, anti-CR3 decreased phagocytosis by approximately 25% ($P < 0.05$) (Fig. 2c). Additionally, blockade with anti-CR3 significantly decreased the number of conidia per macrophage in both cell lines ($P < 0.05$) (Fig. 2d). Anti-CR1 did not have a significant effect on the percentage of phagocytosis and the load of conidia per 100 macrophages. No additive effect was observed when the macrophages were incubated simultaneously with anti-CR1 and anti-CR3 (Fig. 2c and d). These results suggest that opsonization is a complement-dependent process and it is at least partially mediated by CR3.

Effect of α-methyl-D-mannoside (α-MM) on the infection with opsonized *Paracoccidioides brasiliensis* conidia

Since both cell lines showed a high percentage of phagocytosis of nonopsonized *P. brasiliensis* conidia, and mannan is one of the main constituents of the cell wall of *P. brasiliensis* (Kanetsuna *et al.*, 1969; Carbonell *et al.*, 1970; Azuma *et al.*, 1974), we decided to study whether mannose receptor (MR) is involved in phagocytosis of *P. brasiliensis* conidia. For this purpose, macrophages were treated with different concentrations of α-MM, and then infected with opsonized *P. brasiliensis* conidia. Treatment with α-MM inhibited phagocytosis of *P. brasiliensis* (Fig. 3a) and decreased the number of conidia per 100 B10R macrophages, with a peak of inhibition occurring at 5 μg mL^{-1} (Fig. 3b). However, in the case of B10S macrophages only 10 μg mL^{-1} of α-MM

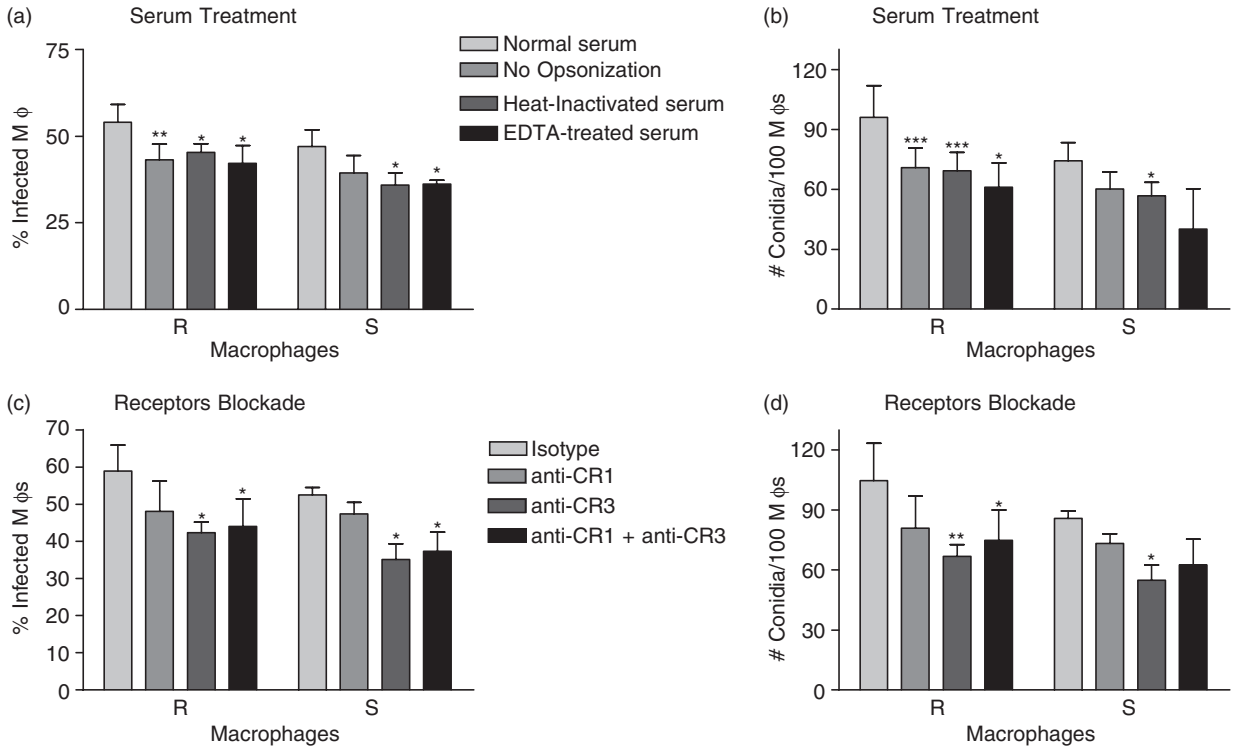

Fig. 2. Effect of complement inactivation and treatment with anti-CR1 and anti-CR3 monoclonal antibodies on phagocytosis of *Paracoccidioides brasiliensis* conidia. Macrophages were infected for 6 h with nonopsonized or opsonized conidia (2 : 1) with normal, heat inactivated or EDTA treated mouse serum. Then the percentage of phagocytosis (a) and the number of conidia per 100 macrophages (b) were determined. To determine the participation of complement receptors, macrophages were incubated with monoclonal anti-CR1, anti-CR3 antibodies or isotype control and were then infected with conidia opsonized with normal mouse serum. The percentage of phagocytosis (c) or the number of conidia by 100 infected macrophages (d) were determined. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

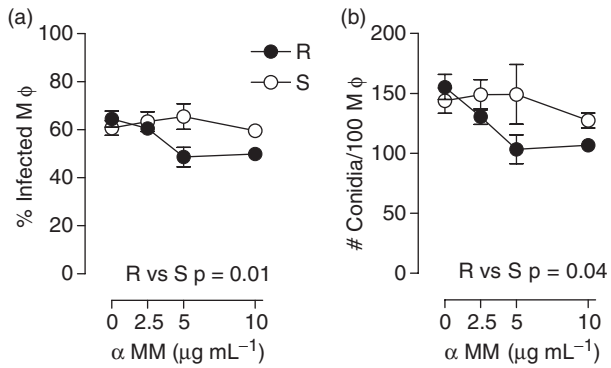

Fig. 3. Effect of α-methyl-D-mannoside (α-MM) on the phagocytosis of *Paracoccidioides brasiliensis* conidia by B10R and B10S macrophages. Macrophages (B10R, closed circles; B10S, open circles) were treated with different doses of α-MM for 1 h and infected for 6 h with opsonized *P. brasiliensis* conidia (2 : 1). The percentage of phagocytosis (a) and the fungal load per cell (b) were determined as described in the Materials and methods. Results were analysed by two-way ANOVA ($n = 3$).

induced reduction in the number of conidia per 100 macrophages. These results clearly show that MR is functionally involved in phagocytosis of *P. brasiliensis* conidia by B10R macrophages at a larger extent than by B10S macrophages that are derived from mice that do not express the Nramp1 protein.

Expression of CR1 (CD35), CR3 (CD11b) and MR on the surface of B10R and B10S macrophages

The results described above suggest that phagocytosis of *P. brasiliensis* conidia is mediated by opsonic and nonopsonic mechanisms. The opsonin-dependent adherence and phagocytosis may be mediated by complement factors and CR3, while the opsonin-independent events may involve MR. Thus, to further explore the relative contribution of CRs and MR, the expression of CR1 (CD35), CR3 (CD11b) and MR were studied by flow cytometry in both macrophage cell lines. CR1 (CD35) was not detectable in either cell line (Figs 4a and b). In contrast, almost all macrophages expressed CR3 (CD11b) (Figs 4a and b); although the level of expression was higher in B10S macrophages that in B10R cells ($P < 0.001$). The percentage of cells that expressed MR (Figs 4c and d), and its level of expression, were higher in B10R macrophages than in B10S ($P < 0.01$ and $P < 0.001$, respectively). These results further support the role of CR3 and

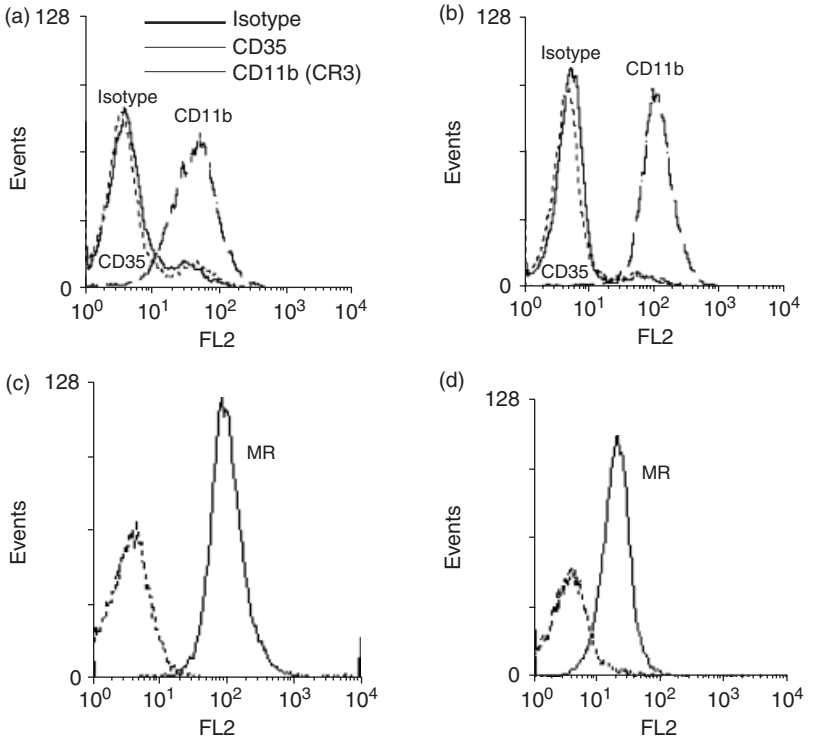

Fig. 4. CR1, CR3 and mannose receptor expression in B10R and B10S macrophages. B10R cells (a), B10S (b) cells were incubated with anti-Fcγ receptor followed by specific anti-CR1 and anti-CR3 or IgG_{2a} isotype control and then stained with goat anti-mouse IgG-FITC. For mannose receptor (MR), B10R (c) and B10S (d) macrophages were stained with PE-labelled mannosylated albumin, and as control with biotin-labelled albumin followed by PE-streptavidin. Fluorescence was determined by flow cytometry by size (FCS) and granularity (SSC), FL1 (FITC) and FL2 (PE). The figure shows histograms of one representative experiment ($n = 3$).

MR in phagocytosis of *P. brasiliensis* conidia by B10R macrophages that express the nonmutated wild type allele of the *Nramp1* gene (*Nramp1*^{Gly169}).

Intracellular conversion of *Paracoccidioides brasiliensis* conidia

The establishment of active *P. brasiliensis* infection requires the conversion of conidia to yeast form (McEwen *et al.*, 1987), Thus, it was important to determine whether both macrophage lines differed in their capacity to inhibit fungal intracellular conversion. Since infected macrophages are exposed *in vivo* to IFN-γ, produced by either NK or T cells, a process known to activate the antifungal activity of infected macrophages (González *et al.*, 2000), it was also relevant to establish if IFN-γ has any differential effect on them. In order to address these questions, the next experiments evaluated the intracellular conversion of *P. brasiliensis* conidia occurring in B10R- and B10S-infected macrophages in the presence of different doses of IFN-γ. After 120 h of infection with opsonized conidia (conidia : macrophage ratio 2 : 1), there was no significant difference observed between the percentage of conversion to yeast forms of the phagocytosed conidia in nonstimulated B10R compared to B10S cells. However, in B10R macrophages treated with IFN-γ the percentage of conidia converted to yeast cells significantly decreased in a dose-dependent way (Fig. 5). On the contrary, in B10S macrophages treated with IFN-γ, the percentage of conidia conversion increased in a dose-dependent manner.

Discussion

The results presented herein show that B10R and B10S macrophages respond differently to the infection with *Paracoccidioides brasiliensis* conidia. A higher percentage of B10R macrophages compared to B10S macrophages phagocytosed both opsonized and nonopsonized conidia. These results were observed irrespective of the dose and time of infection. Furthermore, only a few B10R macrophages were observed without conidia, moreover a vast majority of them had a high number of conidia per macrophage. A significantly higher percentage of B10R cells had 3–4 and ≥ 5 conidia per macrophage, compared to B10S cells, further supporting the hypothesis of the enhanced phagocytic capacity of the B10R macrophages derived from mice expressing the Nramp1 protein (*Nramp1*^{rGly169} *allele*), compared to B10S macrophages derived from mice that do not express the Nramp1 protein (*Nramp1*$^{s\ Asp169}$ *allele*). The difference in phagocytosis of *P. brasiliensis* between B10R and B10S macrophages does not result from a generalized difference in the ability of these macrophages to phagocytose, since no difference was reported in their ability to phagocytose latex beads, *M. smegmatis* (Radzioch *et al.*, 1991) and *M. tuberculosis* H37Rv (Rojas *et al.*, 1997). It is possible that these differences reflect the variation in the mechanisms of adherence/

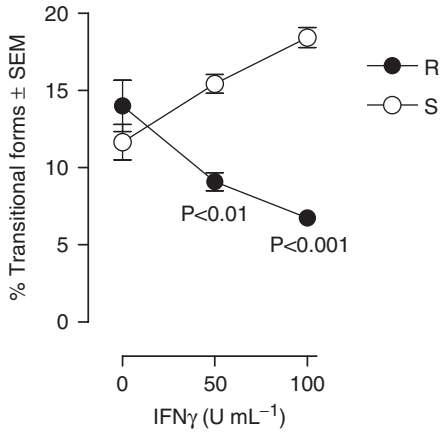

Fig. 5. Effect of IFNγ on the intracellular conversion of *Paracoccidioides brasiliensis* conidia and B10R and B10S macrophages. Macrophages were infected with opsonized conidia at 2 : 1 conidia : macrophage ratio for 120 h, different doses of recombinant murine IFNγ. After incubation, the morphology (conidia, transitional, yeast or multiple budding yeasts cells) of the fungus phagocytosed by B10R (closed symbols) and B10S (open symbols) macrophages was recorded. Results were analyzed by two-way ANOVA.

ingestion required by microorganisms belonging to separate phyla.

Our results demonstrate that the opsonization of conidia, using murine serum, mediated the activation of the complement system leading to CR3-dependent adherence and phagocytosis of the propagules. Serum heat-inactivation and treatment with EDTA, as well as macrophage incubation with monoclonal anti-CR3 antibodies, decreased the phagocytosis to a percentage similar to the one observed without murine serum. Interestingly, anti-CR1 antibodies did not affect the ability of B10R or B10S macrophages to phagocytose conidia. The percentage of inhibition was similar for both cell lines, suggesting that the differences between B10R and B10S macrophages were not due to the expression of complement receptors. This conclusion was also confirmed by finding a similar percentage of B10R and B10S macrophages expressing CR3 protein on their surface. The fluorescence intensity, measured using specific anti-CR3 antibodies, was higher for B10S than B10R cells, demonstrating that that the density of CR3 receptors per cell was higher in macrophages derived from mice that do not express Nramp1 protein. Therefore, the superior ability to phagocytose shown by B10R cells compared to B10S cells can not be attributed to an increase in the CR3 receptors in B10R cells since our results have demonstrated a higher density of CR3 receptors on B10S cells than B10R cells. Neither B10R nor B10S expressed CR1 (CD35), which explains why its blockade did not produce significant changes in phagocytosis.

Our results extend early findings on the importance of complement in phagocytosis of *P. brasiliensis* yeasts (Calich *et al.*, 1979); however, in our experiments we used conidia, the infecting form of *P. brasiliensis*, indicating that both forms of the fungi have cell wall compounds able to activate complement and generate an opsonic activity. Furthermore, our data demonstrate the involvement of CR3 (CD11b), which was not investigated in the pioneer work by Calich *et al.* (1979). However, the CR3 receptor can participate in nonopsonic phagocytosis as well (Ofek *et al.*, 1995). There is evidence that CR3 is able to bind 1–3β glucans and mediate phagocytosis of microorganisms that present this compound on their cell wall (Thornton *et al.*, 1996; Xia *et al.*, 1999) such as *P. brasiliensis* (Kanetsuna *et al.*, 1969; Carbonell *et al.*, 1970; Azuma *et al.*, 1974). Therefore, the CR3 receptor may participate in phagocytosis of *P. brasiliensis* conidia through both opsonic and nonopsonic mechanisms.

Considering that B10R macrophages exhibited a higher percentage of nonopsonic phagocytosis, and higher mannose receptor expression than B10S macrophages, it is possible to attribute these basal differences between the two macrophage cell lines to their diverse phagocytic capacity. The mannose receptor is a C-type lectin able to recognize mannose residues present on the surface of a wide spectrum of microorganisms (bacteria, fungi and protozoa) (Linehan *et al.*, 2000), including *P. brasiliensis* (Kanetsuna *et al.*, 1969; Carbonell *et al.*, 1970; Azuma *et al.*, 1974). Additional evidence regarding the importance of the mannose receptor in the phagocytosis of *P. brasiliensis* conidia by B10R macrophages was obtained by the treatment of macrophages with α-methyl-mannoside that reduced the percentage of phagocytosis.

Since a higher proportion of B10R than B10S macrophages phagocytosed *P. brasiliensis* conidia, the next question was to determine whether both macrophage cell lines differ in their capacity to inhibit the conversion from conidia to yeast. This conversion process is very important in *P. brasiliensis* pathogenicity (McEwen *et al.*, 1987), and it is more efficient when the conidia are located intracellularly both in murine macrophages (Brumer *et al.*, 1989; Cano *et al.*, 1992) and human monocytes/macrophages (Moscardi-Bachi *et al.*, 1993). There was no difference in the conversion of conidia between both cell lines under basal experimental conditions. Interestingly, IFN-γ stimulation of B10R, but not of B10S, macrophages derived from mice that do not express Nramp1 protein, resulted in a dose-dependent decrease in the percentage of conidia to yeast conversion. IFN-γ activates many different macrophages functions, including reactive nitrogen intermediates (RNI) production. It is well documented that RNIs have anti-microbial effect against several types of bacteria and parasites infections (Green & Nacy, 1993; Fang, 1997; MacMicking *et al.*, 1997), including *P. brasiliensis* (González *et al.*, 2000).

Most of the *in vitro* studies on phagocytosis of *P. brasiliensis* have used peritoneal macrophages from BALB/c

strain of mice that carries a mutated form of the *Nramp1* gene and do not express Nramp1 protein (Brumer *et al*., 1989; Cano *et al*., 1992; González *et al*., 2000). Our studies employed immortalized bone marrow B10R and B10S macrophage cell lines, both derived from congenic strains on B10.A genetic background that express the resistant allele of the *Nramp1*^{r} gene or a mutated susceptible allele of the *Nramp1*^{s} gene (null Nramp1 protein expression), respectively. However, it must be noted that the segment of the murine chromosome 1 that differentiate B10R and B10S congenic macrophage lines span at least 13 cM (Schurr *et al*., 1989). Interestingly, within this chromosomal region is located the *susceptibility to tuberculosis 1* (*sst1*) locus (Kramnik *et al*., 2000), which was recently shown to code for the candidate gene *Intracellular pathogen resistance I* (*Ipr1*) that controls the macrophage capacity to limit the multiplication of *Listeria monocytogenes* (Boyartchuk *et al*., 2004) and *M. tuberculosis* (Pan *et al*., 2005). Therefore the potential involvement of *sst1* locus in the genetic susceptibility to *P. brasiliensis* cannot be excluded as no *sst1* knockout mice are available yet. We had chosen this model of resting differentiated macrophages, rather than peritoneal macrophages, which might not represent the best source of macrophages for such studies, since it requires the injections of thioglycollate medium that contains endotoxins into the mice to recruit inflammatory macrophages to peritoneum. Macrophages prestimulated *in vivo*, even with traces of endotoxin, are very likely to have modified the expression of surface receptors. The employed model of immortalized bone-marrow-derived macrophages has been previously very well characterized (Radzioch *et al*., 1991). These macrophages do not express any constitutive levels of inflammatory mediators and were shown to faithfully represent a spectrum of responsiveness and activation observed *in vitro* using differentiated bone marrow macrophages. The results obtained in our studies are concordant with *in vivo* data showing higher ability of mice expressing a wild type (resistant) allele of the *Nramp1* to control *P. brasiliensis* infection. To our knowledge, the importance of CR3 and mannose receptor in the phagocytosis of *P. brasiliensis* has not been previously documented and therefore our findings contribute significantly to the understanding of the mechanism of susceptibility to *P. brasiliensis*. Overall our results might provide new clues useful in developing new treatments that might enhance clearance of *P. brasiliensis* by the infected patients.

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