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Pulmonary Paracoccidioidomycosis in Resistant and Susceptible Mice: Relationship among Progression of Infection, Bronchoalveolar Cell Activation, Cellular Immune Response, and Specific Isotype Patterns

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Using the intraperitoneal route of infection, we demonstrated previously that A/Sn mice are resistant and B10.A mice are susceptible to *Paracoccidioides brasiliensis* infection. Since paracoccidioidomycosis is a deep systemic granulomatous disorder that involves primarily the lungs and then disseminates to other organs and systems, we herein investigated the course of the infection and the resulting immune responses developed by A/Sn and B10.A mice after intratracheal infection with *P. brasiliensis* yeast cells. It was observed that A/Sn mice develop a chronic benign pulmonary-restricted infection, whereas B10.A mice present a chronic progressive disseminated disease. A/Sn animals were able to restrict fungal infection to the lungs despite the increased fungal load at the beginning of the infection. This behavior was associated with low mortality rates, the presence of adequate and persistent delayed-type hypersensitivity reactions, oxidative burst by bronchoalveolar cells, and production of high levels of specific antibodies in which immunoglobulin G2a (IgG2a) and IgG3 isotype titers were significantly higher than those observed in the susceptible mice. In contrast, B10.A animals showed a constant pulmonary fungal load and dissemination to the liver and spleen. This infection pattern resulted in high mortality rates, discrete delayed-type hypersensitivity reactivity, poorly activated or nonactivated bronchoalveolar cells, and production of specific IgG2b isotype titers significantly higher than those observed in the resistant mice at week 4 of infection. Thus, A/Sn and B10.A mice maintain the same resistance patterns as those observed previously with the intraperitoneal route of infection. Furthermore, the obtained results suggest that resistance to paracoccidioidomycosis is associated with T-cell, macrophage, and B-cell activities that are known to be mediated by gamma interferon.

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. Geographically, it is confined to Latin America, with its areas of endemicity extending from Central America to Argentina, and it constitutes one of the most prevalent deep mycoses in this region (42).

The infection can be acquired by the respiratory route (51), probably by inhalation of airborne propagules (28) produced by the fungal mycelial form (6). Most infected subjects develop an asymptomatic pulmonary infection, although some individuals present clinical manifestations giving rise to the different forms of PCM disease, namely, acute, subacute (juvenile type), and chronic (adult type) (31). In most cases, the disease involves primarily the lungs and then disseminates to other organs and systems. Experimental (15, 43) and clinical (2, 34, 35) evidence indicates the relevance of humoral and/or cellular immune response in the pathogenesis and evolution of PCM. Cell-mediated immune response seems to play an important role in resistance to *P. brasiliensis*. Patients with systemic PCM tend to show depressed cellular immune responses compared with those with localized disease (34); furthermore, the most severe forms of the disease are associated with high levels of specific antibodies (11).

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Using a murine model of intraperitoneal (i.p.) PCM, Calich et al. (10) showed that there are significant differences in susceptibility among inbred strains: A/Sn mice were found to be the most resistant, while the B10.A animals were the most susceptible to i.p. *P. brasiliensis* infection. Resistant mice present a low fungal load in several organs and tissues and a tendency to cure, while susceptible animals develop a progressive disease (43).

Studies with F1, F2, and backcross progeny of resistant (A/Sn) and susceptible (B10.A) mice revealed that resistance to PCM is linked to an autosomal dominant gene which we designated the *Pbr* gene (8). Comparisons between the strain distribution of the *Pbr* gene and the genes that control resistance to other experimental infections demonstrated that the *Pbr* gene may be associated with the gene that governs resistance to *Histoplasma capsulatum* (17, 52) and with the closely linked or identical *Lsh*, *Bcg*, and *Ity* genes that confer resistance, respectively, to *Leishmania donovani*, *Mycobacterium bovis*, and *Salmonella typhimurium* (29, 40, 41, 45).

Experimental studies done with other pathogenic fungi revealed that the route of infection influences the immune response profiles of infected animals (32). When A/Sn and B10.A mice were infected with *P. brasiliensis* by the subcutaneous route, differences in resistance to PCM were not observable (9). On the contrary, the resistance patterns of mice infected by the intravenous route were very similar to those observed when infection was done by the i.p. route (9).

The purpose of this work was to verify whether A/Sn and

B10.A mice intratracheally (i.t.) infected with *P. brasiliensis* yeast cells display the same resistance profile observed after i.p. infection and whether an association could be found between the resistance and/or susceptibility to the fungus with bronchoalveolar cell activation and B- and/or T-cell-mediated responses of the host.

MATERIALS AND METHODS

Animals. Groups of 8 to 10 male mice (9 to 11 weeks old) from the susceptible (B10.A) and resistant (A/Sn) strains were used for each period of infection. The animals were bred at the University of São Paulo animal facilities and provided with acidified water and sterilized food.

Fungus. *P. brasiliensis* isolate 18, which is highly virulent (24), was used throughout this study. To ensure the maintenance of its virulence, the isolate was used after three animal passages (25). *P. brasiliensis* 18 yeast cells were then maintained by weekly subcultivation in the semisolid culture medium of Fava Netto (19) at 35°C and used on day 7 of culture. The fungal cells were washed in phosphate-buffered saline (PBS; pH 7.2) and counted in a hemocytometer, and the concentration was adjusted to 20×10^6 fungal cells ml^{-1}. The viability of fungal suspensions, determined with Janus green B vital dye (Merck, Darmstadt, Germany) (4), was always higher than 80%.

Surgical i.t. inoculation. Mice were anesthetized with 10 ml of 2-(2,6-xylidine)-5,6-dihydro-4*H*-1,3-thiazine hydrochloride (Rompun; Bayer of Brazil) per kg (body weight) in an 0.4% solution by the i.p. route. After 10 min, the animals were injected i.p. with 10 ml of chloral hydrate (Reagen; Quimibrás, Ind. Químicas, Rio de Janeiro, Brazil) per kg (body weight) in a 2.5% solution. Animals were restrained on a small board and infected with 10^6 fungal cells (*P. brasiliensis* 18) by surgical i.t. inoculation. For this purpose, a small incision was made through the skin over the trachea, and the underlying tissue was separated; a 30-gauge needle attached to a tuberculin syringe was inserted into and parallel with the trachea, and 50 μl of inoculum was dispensed into the lungs. The skin was then sutured, and the mice were allowed to recover under a heat lamp.

Mortality rates. Mortality studies were done with groups of 22 to 24 A/Sn and B10.A mice inoculated i.t. with 10^6 fungal cells and 24 A/Sn and B10.A control mice inoculated with PBS. Deaths were registered daily for a 400-day period, and the median survival days postinfection were calculated.

Assay for organ CFU. The determination of the number of viable microorganisms in different organs of the infected mice was done by CFU counts. At various times postinfection (1, 2, 4, 8, 12, and 16 weeks), eight infected animals of each mouse strain were sacrificed and their lungs, livers, and spleens were removed aseptically, weighed, and homogenized in 5 ml of sterile PBS with a tissue grinder. The cellular suspensions were washed three times in PBS, and the final pellets were resuspended in 1 ml of PBS. Aliquots of 100 μl of each homogenate were plated on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) containing 4% (vol/vol) normal horse serum (Inst. Butantan, São Paulo, Brazil) and 5% *P. brasiliensis* 192 culture filtrate, the latter constituting the source of growth-promoting factor (16, 44). Dilutions were made in sterile PBS when necessary. Plates were incubated at 35°C, and colonies were counted daily until no increase in counts was observed. The numbers ($\log_{10}$) of viable *P. brasiliensis* colonies per gram of tissue are expressed as the mean ± standard error.

Delayed-type hypersensitivity (DTH) assay. The DTH reactions of the same animals used for the CFU determinations were evaluated by the footpad test under the conditions described by Fazioli et al. (21). Briefly, 24 h before sacrificing the animals, the footpad thicknesses were measured with a dial caliper (precision, 0.01 mm; Mitutoyo Corporation, Tokyo, Japan); immediately after, the mice were challenged by injection of 25 μl of the antigen of Fava Netto et al. (20) derived from *P. brasiliensis* isolate 18 (protein concentration, 310 μg ml^{-1}). The footpad thickness was measured 24 h later, and the swelling was expressed in millimeters. Noninfected mice subjected to the footpad test were used as controls.

H_2O_2 production by bronchoalveolar cells. The H_2O_2 production by bronchoalveolar cells was determined by the horseradish peroxidase-dependent phenol red oxidation method (39) and slightly modified by us as follows. The cells obtained from each individual mouse after bronchoalveolar lavage by repeated injection of 1 ml of sterile PBS (final volume, 5 ml) were adjusted to 2×10^6 cells per ml with freshly prepared phenol red solution that contained Dulbecco's PBS, 5.0 mM dextrose, 50 μg of horseradish peroxidase type II per ml, and 0.28 mM phenol red (all reagents from Sigma Chemical Co., St Louis, Mo.). Volumes (100 μl) of the cell suspensions were plated onto each well of 96-well flat-bottom tissue culture plates (Costar, Cambridge, Mass.). In some wells, 10 ng of phorbol myristate acetate (PMA; Sigma) or 2×10^5 heat-killed yeast cells of *P. brasiliensis* were used as a triggering agent. A standard curve was constructed with H_2O_2 solutions at final concentrations of 0.5, 1, 2, or 4 nmol per 100 μl of phenol red solution. The plates were incubated at 37°C for 1 h, and the reactions were stopped by adding 10 μl of 1 N NaOH. The A_{610} was measured in an enzyme-linked immunosorbent assay (ELISA) reader (M_r, 5,000; Dynatech Laboratories Inc., Chantilly, Va.). The results were expressed as nanomoles of H_2O_2 per 2×10^5 cells.

Specific antibody levels. Specific antibody levels were measured by the classical ELISA with a cell-free antigen preparation as described previously (12). This antigen preparation was made with a pool of different *P. brasiliensis* isolates (B339, 265, and 18). Briefly, the antigen solution (1:100) was dispensed into each well of a 96-well flat-bottom plate (model P-0011; Difco) and incubated for 1 h at 37°C and overnight at 4°C. The wells were washed and blocked with 0.5% gelatin (Difco) by incubation for 1 h at 37°C. After three washes, serial dilutions of each serum sample were added to the well. After incubation for 1 h at 37°C, goat anti-mouse total immunoglobulins (Ig), immunoglobulin G1 (IgG1), IgG2b (1:4,000), and IgM, IgG2a, and IgG3 (1:1,000; Southern Biotechnology Associates, Inc., Birmingham, Ala.) were added to the wells and the plates were incubated for 1 h at 37°C. Rabbit anti-goat IgG conjugated to horseradish peroxidase (1:8,000; Southern Biotechnology Associates) was dispensed into each well, and the plates were incubated for 1 h at 37°C. The optimal dilutions of *P. brasiliensis* antigen, antisera, and conjugate were determined initially by block titration. The reaction was developed with a substrate solution containing 3.7 mM *o*-phenylenediamine (Merck) and 3.52 mM hydrogen peroxide (H_2O_2; Merck) in 0.05 M citrate buffer (pH 5.0), and plates were kept in the dark at room temperature for 15 min. The reaction was stopped by adding 4 N sulfuric acid to each well. The optical densities were measured at 490 nm with an ELISA reader (M_r, 5,000; Dynatech Laboratories). The average of the optical densities obtained with sera from control mice (PBS inoculated), diluted 1:20, was considered the cutoff for each respective isotype. The optical densities for each dilution of experimental sera were compared with the control values. The titer for each sample was expressed as the reciprocal of the highest dilution which presented an absorbance value higher than the cutoff value.

Statistical analysis. The number of CFU per gram of tissue and the antibody titers were transformed into logarithmic units before statistical analysis. A two-way analysis of variance followed by multiple comparisons by the Tukey method was used to compare the two mouse strains at the six times postinfection with regards to the fungal dissemination to each organ examined, the DTH responses, and the antibody titer.

The DTH responses of infected animals that were higher than the upper limit of the 95% confidence interval of control mice (mean + 2 standard deviations) were considered significant.

The survival times of infected and control B10.A and A/Sn animals were analyzed by a nonparametric two-factor analysis of variance and then by multiple comparisons by the Dunn method.

Studies of the correlation of the numbers of CFU in the distinct organs, the DTH reactions, and the specific Ig titers were done by Spearman's multiple correlation method (53).

A P of <0.05 was always considered significant.

RESULTS

Mortality rates. The median survival times observed in infected B10.A and A/Sn mice were 181 and 393 days, respectively, whereas 95% of control A/Sn and B10.A mice survived more than 400 days. Infected B10.A mice presented significantly lower survival times than both their control counterparts and infected A/Sn animals (Fig. 1). The earlier and faster mortality of B10.A mice, compared with that of A/Sn animals, demonstrated that when the i.t. route of infection is used, B10.A mice are more susceptible to the fungus than the A/Sn strain.

Pulmonary infection and extrapulmonary dissemination. The evolution of the disease in B10.A and A/Sn mice was monitored by CFU counts in the lungs, liver, and spleen at different postinfection periods (24 h and 1, 2, 4, 8, 12, and 16 weeks). As shown in Fig. 2, equivalent numbers of CFU were found in the lungs of both mouse strains at 24 h postinfection. At week 1 after infection, both mouse strains presented a significant pulmonary clearance compared with the CFU counts at 24 h. However, from week 1 to week 8, A/Sn mice presented progressively increased CFU counts in the lungs. Conversely, the number of CFU in the lungs of B10.A mice was similar to that verified at week 1 postinfection and remained constant for all periods analyzed. Furthermore, from weeks 1 to 8 after infection, the numbers of viable yeast cells in pulmonary tissues of B10.A animals were significantly lower than those observed in A/Sn mice. At weeks 12 and 16, A/Sn animals presented a significant reduction in numbers of pulmonary CFU; these numbers were similar to the values observed in the B10.A counterparts. An absence of fungal dis-

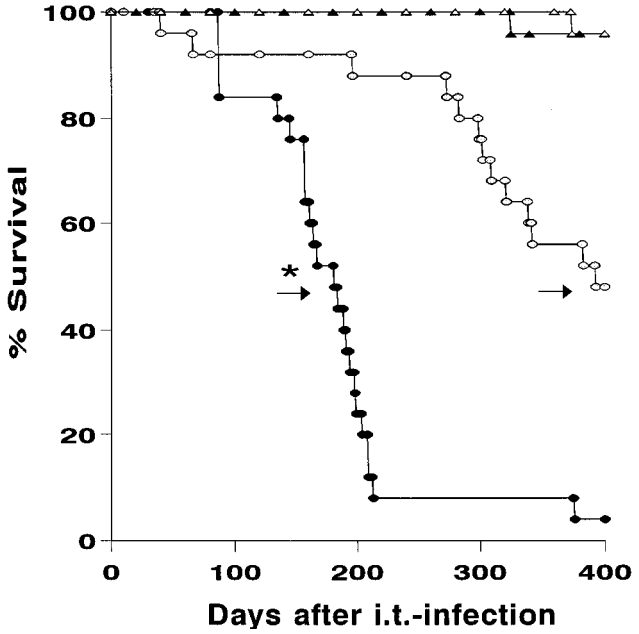

FIG. 1. Survival percentages of A/Sn (○) and B10.A (●) mice ($n = 22$ to 24) observed over 400 days after i.t. infection of mice with 10^6 yeast cells of *P. brasiliensis* isolate 18. Control animals (A/Sn [△] and B10.A [▲]; $n = 24$ for each mouse strain) were inoculated with PBS. Arrows indicate median survival times. *, significantly different ($P < 0.05$) from infected A/Sn mice.

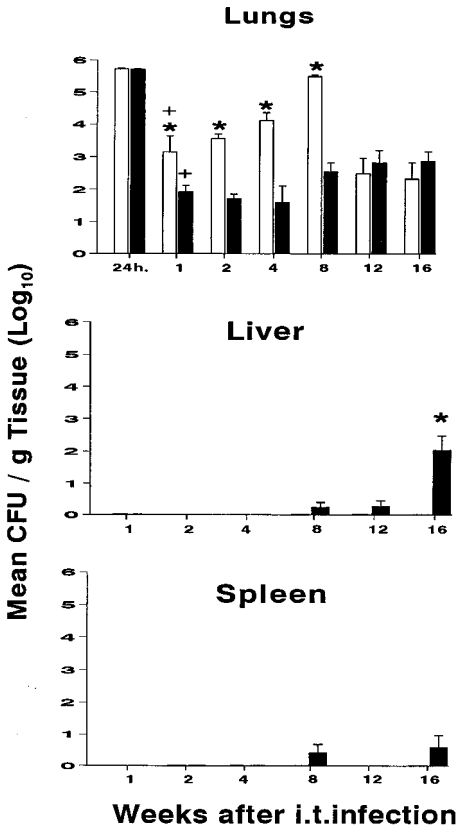

FIG. 2. Time course of pulmonary infection and dissemination to liver and spleen of resistant A/Sn (□) and susceptible B10.A (■) mice inoculated i.t. with 10^6 *P. brasiliensis* isolate 18 yeast cells. Each bar represents the mean of six to eight animals. Range bars represent standard errors of the means. *, significantly different ($P < 0.05$) between strains; +, significantly different ($P < 0.05$) from 24-h counterparts.

semination to the liver and spleen was observed in A/Sn mice. In contrast, B10.A mice presented viable yeast cells in the liver and spleen from week 8 postinfection on (Fig. 2).

Specific cellular immunity. The development of DTH responses to *P. brasiliensis* was examined at various times (1, 2, 4, 8, 12, and 16 weeks) after fungal inoculation of A/Sn and B10.A mice. Both mouse strains presented significant DTH responses at all assayed datum points compared with those of control animals. However, the DTH response profiles in the course of the infection of B10.A and A/Sn mice were quite distinct, as shown in Fig. 3. A/Sn mice presented significantly higher reactions than B10.A animals at all assayed datum points, except at week 2, when B10.A mice presented a significantly higher DTH response.

Activity of bronchoalveolar cells. The activity of bronchoalveolar cells from infected A/Sn and B10.A mice was determined by their capacity of H_2O_2 release in spontaneous conditions and after in vitro stimulation with PMA or heat-killed *P. brasiliensis* yeast cells (Fig. 4). Spontaneous H_2O_2 release was observed only with cells of infected A/Sn mice, from week 8 postinfection on; at week 8, the cells produced significantly higher H_2O_2 levels than those of B10.A animals. The in vitro stimulation with PMA increased the H_2O_2 production by cells from infected A/Sn mice at weeks 1, 8, 12, and 16. On the other hand, bronchoalveolar cells from infected B10.A mice maintained basal levels of H_2O_2 both in spontaneous conditions and after PMA stimulation. Significant differences between the two mouse strains regarding the H_2O_2 release triggered by PMA were found from week 8 postinfection on. When heat-killed fungal cells were used as an in vitro stimulus, the infected A/Sn mice produced H_2O_2 during all studied periods, while B10.A animals released discrete levels of H_2O_2 at weeks 1, 4, and 8 postinfection. The H_2O_2 production by bronchoalveolar cells stimulated by heat-killed fungal cells was significantly higher in A/Sn mice than in B10.A mice at weeks 8, 12, and 16 postinfection.

Specific humoral response. Specific antibodies to *P. brasiliensis* were determined as total Ig, IgM, IgG1, IgG2a, IgG2b, and IgG3 in the sera of both studied mouse strains. The results obtained are presented in Fig. 5. The specific antibody response as measured by anti-total Ig occurred earlier in B10.A (at week 2 postinfection) than in A/Sn mice. At week 4, susceptible animals presented significantly higher total Ig than the resistant mice. However, at week 8, the total Ig titers of A/Sn animals increased abruptly and the antibody levels in these mice were significantly higher than those found in B10.A mice. From this time on, both mouse strains produced high and similar levels of antibodies. IgM production was relatively low in both mouse strains at all analyzed periods, although at week 8 postinfection, A/Sn mice presented significantly higher IgM production than the B10.A counterparts. Detectable levels of IgG1 antibodies were found as early as 2 weeks postinfection in B10.A animals; subsequently, the antibody production increased in both mouse strains, and at week 12, the resistant A/Sn mice presented significantly higher IgG1 levels than the B10.A animals. The IgG2a isotype was produced at later stages of infection (from week 8 on) in both mouse strains, and the titers were always significantly higher in A/Sn mice than in B10.A mice. Specific IgG2b levels were detectable in the sera of B10.A mice from week 2 of infection on and in A/Sn animals after week 8; B10.A presented significantly increased IgG2b levels at week 4 postinfection compared with those of the A/Sn counterpart, but at later periods (8, 12, and 16 weeks), both

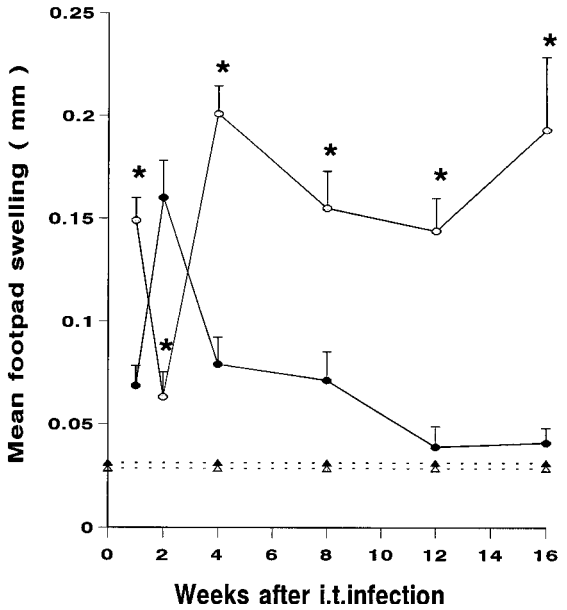

FIG. 3. Mean DTH reactions in A/Sn (○) and B10.A (●) mice infected i.t. with 10^6 *P. brasiliensis* isolate 18 yeast cells (n = 6 to 8). Range bars represent standard errors of the means. The horizontal dotted lines denote means + 2 standard deviations (95% confidence interval) of noninfected A/Sn (△) and B10.A (▲) mice subjected to the footpad test (n = 39). *, significantly different ($P < 0.05$) from B10.A mice.

mouse strains produced similar levels of this isotype. The IgG3 isotype, like IgG2a, was produced at later stages of the disease (weeks 8, 12, and 16), and the titers were always significantly higher in A/Sn than in B10.A mice.

Correlations among progression of infection, cellular immune response, and isotype patterns. To evaluate possible relationships among the different parameters measured in the same animal, correlation coefficients (r) were calculated with data obtained in B10.A mice and combining all assay times after infection. Correlation studies with A/Sn mice were hampered because of the total absence of dissemination in diverse organs at variable times postinfection.

The number of CFU in the liver correlated with the counts in the lungs (r = 0.50; P = 0.001) and spleen (r = 0.42; P = 0.006). The total Ig titers were also correlated with the number of viable yeast cells in all studied organs ($0.001 < P < 0.02$). However, the intensity of DTH reactions was inversely correlated with the total antibody titers ($r = -0.47$; P = 0.002) and with the number of CFU found in the lungs ($r = -0.44$; P = 0.005) and liver (r = 0.30; P = 0.050). Furthermore, the levels of all studied isotypes were correlated positively with the total Ig titers (r ranging from 0.57 for IgG3 to 0.95 for IgG2b).

DISCUSSION

To understand the immune mechanisms operating during infections, it is highly desirable to establish the resistance patterns and to characterize the associated immune responses with experimental models that mimic the natural disease. With this concept in mind and considering that PCM is acquired by the respiratory route (42), a time course study of the cellular and humoral immune responses and an evaluation of the bronchoalveolar cell activation were done in the present study with two inbred mouse strains infected i.t. with a highly virulent *P. brasiliensis* isolate, isolate 18. Although yeast forms (which are not the natural propagules of this organism) were used, a model that very much resembles the polar forms of the human disease was obtained.

The longer survival times of i.t. infected A/Sn mice, compared with the mortality rates of B10.A animals, demonstrated that the resistance pattern after i.t. infection of these animals is similar to those observed previously when the i.p. (10) and intravenous routes of infection (9) were used.

The progress of the infection of A/Sn and B10.A mice monitored by organ cultures revealed that the fungal load increased progressively in the lungs of A/Sn mice until week 8 postinfection, decreasing sharply thereafter, but *P. brasiliensis* 18 dissemination to other organs could never be found. On the contrary, B10.A animals maintained the same CFU counts throughout the infection, but fungal dissemination to the liver and spleen was evident. This pattern of dissemination is similar to that obtained after *P. brasiliensis* 18 i.p. infection of B10.A and A/Sn mice (43). In both pulmonary and i.p. infection, a high level of dissemination of the yeast cells to various organs

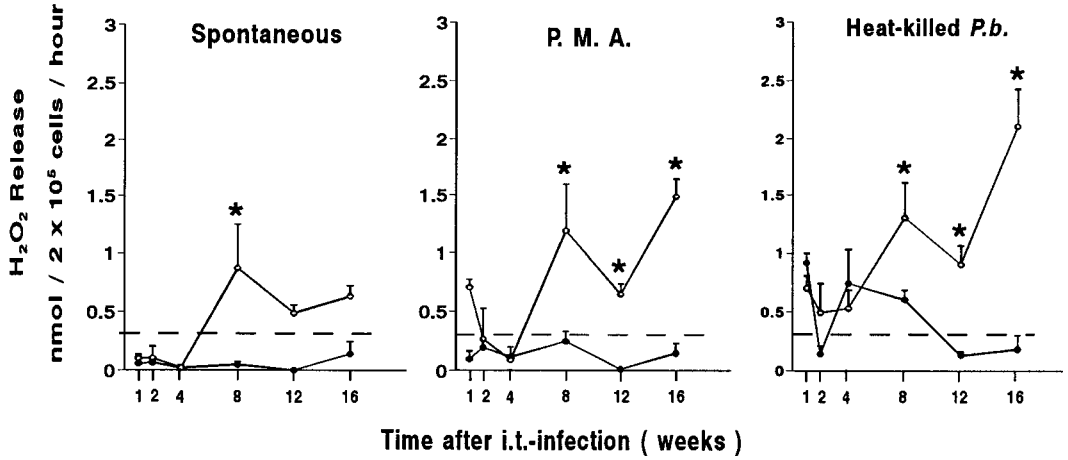

FIG. 4. H_2O_2-generating activity of bronchoalveolar cells in A/Sn (○) and B10.A (●) mice infected i.t. with 10^6 *P. brasiliensis* isolate 18 yeast cells. The H_2O_2 release was measured as spontaneous, after PMA in vitro stimulation, and after heat-killed *P. brasiliensis* (P.b.) in vitro stimulation. The horizontal dotted line denotes significant H_2O_2 production (0.3 nmol of H_2O_2). Each experiment was done with pooled cells from three animals, and the means ± standard errors of the means of five experiments are represented. *, significantly different ($P < 0.05$) from B10.A mice.

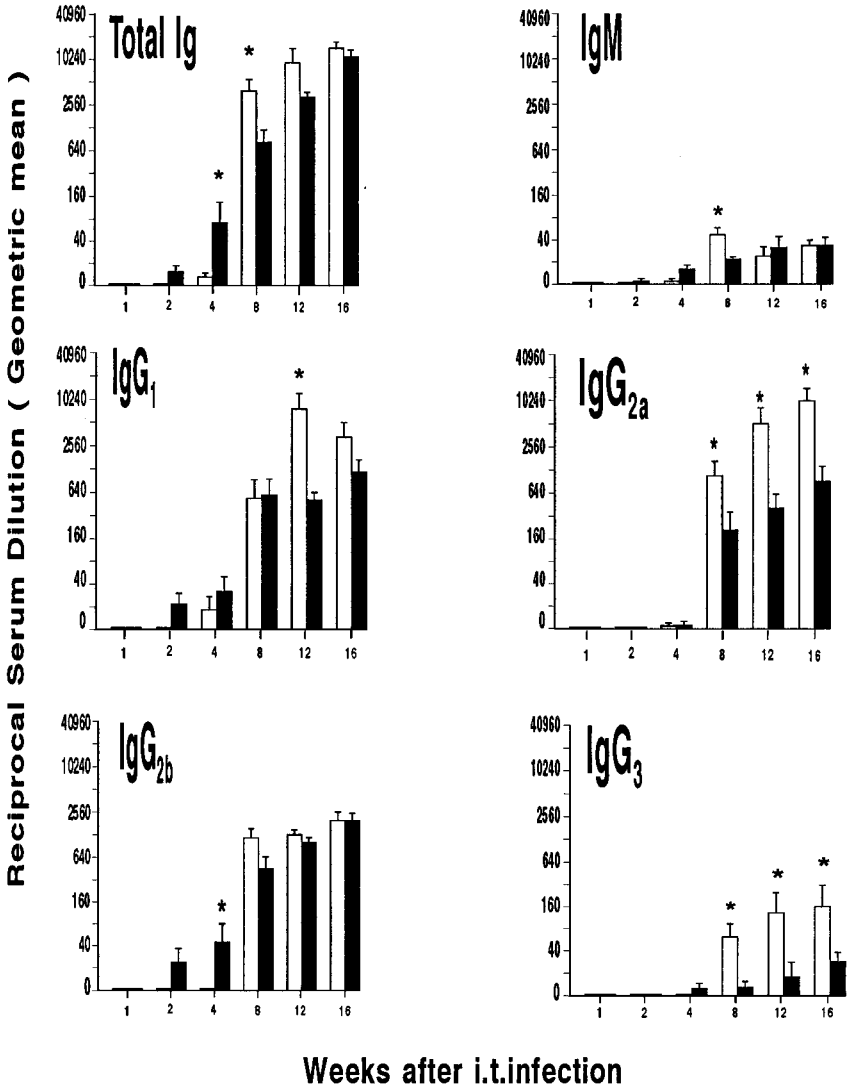

FIG. 5. Specific antibody levels (total Ig, IgM, IgG1, IgG2a, IgG2b, and IgG3) in A/Sn (□) and B10.A (■) mice infected i.t. with 10^6 *P. brasiliensis* isolate 18 yeast cells. Each bar represents the geometric mean of serum titers of 6 to 8 animals. Range bars represent standard errors of the means. *, significant differences ($P < 0.05$) between the mouse strains.

was found in susceptible animals but not in the resistant ones. These results suggest that resistance to *P. brasiliensis* infection is determined mainly by the ability of the host to restrict fungal dissemination rather than control fungal growth at the primary site of infection. The relatively higher fungal load observed in the lungs of resistant animals at the initial phases of the disease (4 and 8 weeks) in both experimental models also demonstrated that resistance to *P. brasiliensis* is not linked to a natural capacity to clear the pathogen load from the lungs, but the acquired immunity lately developed by the hosts could be the main operating factor. Our results are parallel to those reported by Orme and Collins (38), who used a murine model of infection with *Mycobacterium bovis*. These authors verified that the *Bcg* gene activity could be inferred from the growth of *M. bovis* BCG Montreal in the spleen but not in the lungs of inbred strains of mice and that the control of the infection was associated with the generation of acquired resistance of the hosts. Despite the lack of association between the initial fungal clearance and resistance to *P. brasiliensis* infection, the divergent disease outcome found in B10.A and A/Sn mice could be due to different patterns of initially produced cytokines which would induce protective immune responses in A/Sn animals and a nonefficient immunity in B10.A animals.

Extrapulmonary dissemination to the liver and spleen was also reported in other experimental pulmonary PCM models such as that with BALB/c mice intranasally infected with yeast cells (14) or conidia (28) and that with Syrian hamsters inoculated i.t. with yeast cells (48). As observed herein, these authors verified that the dissemination to extrapulmonary tissues occurred at advanced stages of the infection, indicating a chronic process. It is noteworthy that hepatic involvement is also relatively frequent (33%) in chronic human PCM (27). Thus, our model seems to mimic the human disease and indicates that the i.t. infections of A/Sn and B10.A mice with *P. brasiliensis* yeast cells parallel the chronic benign pulmonary restrictive and the chronic progressive disseminated forms of PCM, respectively.

It is well established that cell-mediated immune responses are impaired in human (30, 34, 36) and experimental (15, 21, 43) PCM as well as in some other chronic disseminated fungal

infections (1, 23). In our experimental pulmonary model, the specific cellular immune response, measured in vivo by the DTH responses, was developed efficiently by A/Sn mice from week 4 postinfection on. Conversely, B10.A animals presented an evident DTH response at week 2 of infection, but their reactions were discrete at subsequent stages. The observed DTH patterns in the course of the infection resembled those observed by Fazioli et al. (21) and Singer-Vermes et al. (43) using the i.p. PCM model, where susceptible animals (B10.A) developed anergy in DTH responses and resistant mice presented an evident and persistent cutaneous reactivity. The significant inverse correlations observed between the numbers of CFU in the lungs and liver and the DTH responses of i.p. and i.t. infected B10.A animals suggest that the progression and dissemination of infection may determine a low and perhaps inefficient cellular response in these mice. The cellular immune responses observed at most assay times in A/Sn mice seem to be, in this model, associated with efficient resistance to *P. brasiliensis.*

Bronchoalveolar cells of i.t. infected A/Sn mice were able to release significant amounts of H_2O_2 after in vitro stimulation with both of the triggering agents used (PMA and heat-killed fungal cells); this activity was coincident with the pulmonary fungal load decrease. Since no dissemination to the liver and spleen was observed, it can be inferred that fungicidal mechanisms were operating in A/Sn mouse lungs after 8 weeks of infection. It has been shown previously that gamma interferon (IFN-γ)-activated macrophages are able to kill *P. brasiliensis* yeast cells, although this activity was independent of oxygen products (5). Therefore, high H_2O_2 production by A/Sn macrophages can be interpreted only as a phenotypic marker of resistance and not as an active mechanism of fungal killing. The low levels of H_2O_2 production associated with a constant number of pulmonary *P. brasiliensis* CFU and the dissemination to the liver and spleen found in B10.A mice allow us to infer that the acquired immunity developed by these animals may not be sufficient to activate fungus-killing mechanisms. When activation of peritoneal macrophages of mice infected i.p. with *P. brasiliensis* 18 was evaluated, expressive macrophage activation in A/Sn mice and impairment of this function in B10.A animals were also verified (49). It is well known that IFN-γ is the major macrophage-activating lymphokine (37), whereas interleukin-4 (IL-4) (26) and transforming growth factor β (50) as well as other regulatory products participate in the suppression of the respiratory burst of these cells. Thus, we can suppose that macrophage activation in resistant and susceptible animals is under the regulation of different cytokines. In agreement with this hypothesis, it was verified previously (7) that *P. brasiliensis* i.p. infection induces a sustained major histocompatibility complex class II expression by macrophages from resistant mice and a transient Ia expression by peritoneal macrophages from susceptible animals. Accordingly, Cao et al. (13) have shown previously that persistent and transient Ia expression are regulated by IFN-γ and IL-4, respectively.

Considering the specific humoral immune responses, it was clear that the isotype profiles of specific anti-*P. brasiliensis* antibodies produced by B10.A and A/Sn mice are quite distinct. A/Sn mice produced statistically significant higher titers of IgG2a and IgG3 isotypes when compared with those of the susceptible mice, and the antibodies produced by B10.A animals were equally distributed among the IgG1, IgG2a, and IgG2b subclasses. It is noteworthy that IgG1 and IgG2b are produced much earlier than IgG2a and IgG3 in B10.A mice. A/Sn mice, however, produced high titers of the four isotypes at later time points. The influence of different cytokines on antibody production and isotype expression has been well documented (18, 22, 47). IFN-γ is the major inducer of a switch to IgG2a secretion (3), whereas IL-4 has been associated with isotype switching to IgG1 (mouse) and transforming growth factor β functions as an important IgA and IgG2b switch factor. In addition, IFN-γ has been shown to induce IgG3 secretion in response to T-cell-independent type 2 antigens (46). The data presented here are consistent with a predominant IFN-γ influence on B-cell activation of resistant animals, mainly at the late phases of infection. Other cytokines (IL-4 and, possibly, transforming growth factor β) appear to be the main B-cell regulators of susceptible mice at the initial stages of the infection. Thus, our data suggest that a TH2 response may be dominant in B10.A mice during the early phase of infection, although these animals can also mount a late TH1 type of response. In A/Sn mice, the influences of both TH subsets are present, but at late phases of infection, TH1 cells may predominate.

Taken together, our results favor the idea that susceptibility to *P. brasiliensis* pulmonary infection is associated with acquired immune mechanisms that allow fungal dissemination to other organs and disease progression. This type of immune response fails to efficiently activate pulmonary macrophages, induces low levels of DTH reactivity, and stimulates the production of IgG1-, IgG2b-, and IgG2a-specific antibodies. On the contrary, resistance is linked to evident DTH responses, pulmonary macrophage activation, and antibody response in which IgG2a and IgG3 titers were always significantly higher than those observed in B10.A mice. Since all these activities are regulated mainly by IFN-γ, it is tempting to speculate that resistance to pulmonary PCM is under the control of the TH1 subset of T lymphocytes, which was shown previously to preferentially secrete IL-2 and IFN-γ (33). Further studies on the cytokines produced in the course of experimental *P. brasiliensis* infection and on the effects of in vivo inhibition of IFN-γ and IL-4 by monoclonal antibodies are being performed in our laboratory, and the results will soon become known.

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