Immunity to *Paracoccidioides brasiliensis* infection

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Introduction

The pioneer researchers on paracoccidioidomycosis (PCM) had the merit of recognizing that the various clinical forms of this disease were associated with different patterns of immune response. Thus, the severe forms of the disease, with intense involvement of various organs with progressive lesions, leading to death, were accompanied by gradual loss of specific cellular immune responses and high titres of specific antibodies. The mild forms of the disease, presenting few localized lesions, leading to healing, were parallel to maintained cellular immune responses and low levels of specific antibodies (Fava Netto, 1955; Mendes and Raphael, 1971). Subjects exposed to *Paracoccidioides brasiliensis* propagules usually develop asymptomatic infection, indicating that they are resistant hosts. However, some individuals are susceptible to this fungal agent and develop overt PCM (reviewed by Franco, 1987). The genetic basis of resistance was indicated by the finding of more severe clinical forms developed by Japanese individuals (Lacaz, 1956).

The isogenic murine model of PCM

Since the dawn of research in PCM, several authors have established animal models, some of which were indeed very useful (reviewed by Coelho *et al.*, 1994), but they will not be referred to here. Our group introduced the use of inbred mouse strains for these studies and detected significantly varying patterns of susceptibility between them. The A strains (A/SN, A/J) were found to be the most resistant, while the B10 strains (B10.A, B10D2/nSn, B10D2/oSn) were the most susceptible, as evaluated by mortality data. The H-2 region of the major histocompatibility complex (MHC) did not influence the susceptibility pattern, since the A/SN and B10.A strains, which share the same H-2^a haplotype were, respectively, resistant and susceptible to *P. brasiliensis* (Calich *et al.*, 1985). The studies also suggested that the presence of the final complement component C5 was not decisive in PCM, since the strains resistant to *P. brasiliensis* are C5-deficient and the infection in the congenic pair B10D2/oSn (C5-deficient) and B10D2/nSn (C5-normal) led to similar disease outcome (Burger *et al.*, 1985). Comparing the distribution of susceptibility and resistance patterns to *P. brasiliensis* within the examined mouse strains, similarities with histoplasmosis and coccidioidomycosis were found, but the more striking observation was that the profiles of susceptibility and resistance observed in PCM are very close to those verified for the pathogens *Salmonella typhimurium, Mycobacterium tuberculosis,* and *Leishmania donovani* (Skamene *et al.*, 1982). As is now well documented, the initial resistance to these intracellular pathogens is governed by an autosomal dominant gene *(Ity/Bcg/Lsh)* with pleiotropic effects, expressed in macrophages and controlling the phagocytic and microbicidal ability of these cells (Vidal *et al.*, 1993). Genetic studies performed by our group with the mouse strains presenting polar behaviours after intraperitoneal (i.p.) infection with *P. brasiliensis* have shown that also in this model, there is an autosomal dominant gene controlling resistance to infection (Calich *et al.*, 1987). Therefore, studies employing congenic strains to the *Bcg* gene would be of interest in murine PCM.

We quantified the viable *P. brasiliensis* yeasts that disseminated to the omentum, pancreas, spleen, liver and lungs of susceptible (B10.A) and resistant (A/Sn) mice in the course of i.p. infection. The fungal load in all these organs was higher in the susceptible strain, and increased as the disease evolved, while in the resistant mice fungal counts were lower with a tendency to fungal elimination (Singer-Vermes *et al.*, 1993). The levels of antigenaemia increased as the disease progressed in the susceptible mice, whereas in the resistant mice they oscillated at low levels during the whole course of the infection (Garcia *et al.*, 1997). In the resistant mice,

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the lesions were either residual or well-encapsulated, presenting abundant polymorphonuclear neutrophils (PMNs) and macrophages in areas of massive fungal destruction, indicating the control of the infection. In the susceptible mice, on the other hand, active disease was evidenced by the presence of increasing numbers of multiple lesions with little encapsulation and no evidence of fungal destruction (Xidieh *et al.*, 1997).

As the natural route of infection by *P. brasiliensis* is probably by the inhalation of its propagula and the literature refers to marked alterations of behaviour upon changing the route of inoculation of various infecting agents, a pulmonary model of murine PCM was developed. The susceptibility and resistance patterns observed in the i.p. model were maintained, as reflected both by the higher mortality rates of the B10.A mice when compared to the A/Sn ones, and by the pattern of fungal dissemination developed (Cano *et al.*, 1995). The susceptible mice were not able to restrain the infection to the lungs, allowing dissemination to the liver and spleen after two months of infection and thus developing a chronic disseminated form of the disease; in the resistant mice, on the other hand, no dissemination was observed, characterizing a pulmonary-restricted chronic disease. Within the lungs, however, rather unexpectedly, after an initial clearance in comparable levels in both strains, the numbers of viable yeasts present in the resistant strain were higher than those in the susceptible mice, until the second month of infection. This phenomenon is similar to that described by Orme and Collins (1984), who have reported that the activity of the *Bcg* gene was not expressed by pulmonary macrophages, since resistant animals allowed higher bacterial growth in the lungs. In our model, despite the higher pulmonary fungal loads, the better disease outcome shown by A/Sn mice was associated with the development of an acquired immune response which favour cellular immunity and macrophages activation (Cano *et al.*, 1995).

Phagocytes and their products in the isogenic murine model of PCM

Peritoneal macrophages from A/Sn mice express high levels of the MHC class II antigens persistently upon *in vitro* cultivation, whereas macrophages from B10.A mice express those antigens only transiently (Calich *et al.*, 1994) in a way similar to what has been reported for macrophages from mice resistant and susceptible to *Mycobacterium bovis* infection (Johnsohn and Zwilling, 1985). In this context, the macrophages originating from resistant mice could be expected to be more effective in presenting antigens than those obtained from susceptible mice. This is not the case, however. We compared the effectiveness of splenocytes from normal mice of either strain as antigen-presenting cells (APC) by taking advantage of the fact that these two mouse strains share the same H-2 haplotype. By interchanging the two APCs, we found that both APCs were able to efficiently present *P. brasiliensis* antigens to immunocompetent cells (Sbravate *et al.*, 1995). These results suggest that susceptibility seems not to be associated with an inherent defect of splenic adherent APC, but more probably to different activation pathways further developed in the course of the infection. *In vivo* impairment of macrophage functions by blockade with carbon prior to infection rendered both resistant and susceptible mice considerably more susceptible to infection, as demonstrated by an increase in the mortality rate and dissemination of the lesions (Kashino *et al.*, 1995). These data show the important role played by macrophages in the disease outcome of both mouse strains.

Macrophages from resistant mice produce more hydrogen peroxide than those from susceptible mice, independently if they were bronchoalveolar cells, analysed after i.t. infection (Cano *et al.*, 1995), or peritoneal macrophages, studied after i.p. infection (Calich *et al.*, 1988). It must be kept in mind, however, that the actual killing of *P. brasiliensis* cells is not dependent on products of the oxidative burst (Brummer, 1994), and the higher H_2O_2 production by macrophages from resistant mice must be regarded as indicators of state of activation. Indeed, elegant studies have shown that IFNγ-activated macrophages present improved fungistatic/fungicidal ability, which does not correlate with the production of oxygen metabolites (reviewed by Brummer, 1994).

The oxidative burst of another professional phagocyte, the PMN, was also studied. The production of H_2O_2 by PMNs from infected A/J mice increased during the infection, whereas that of B10.A PMNs remained at the same low levels. Moreover, the production of superoxide anion remained unaltered in resistant mice, whereas it decreased in susceptible ones, indicating that PMNs from B10.A mice lose their ability to produce this substance during the infection. Such different states of activation were parallel to a higher efficiency of PMNs from resistant mice in directly killing *P. brasiliensis* than those from susceptible animals (Meloni-Bruneri *et al.*, 1996). These data, analysed together with the presence of large numbers of PMN close to *P. brasiliensis* yeasts in various stages of lysis in the granulomatous lesions, point towards an important role of this cell population in the control of paracoccidioidomycotic infection.

The production of some of the mediators of natural immunity was studied in the course of i.p. infection. TNFα was produced in low levels by both

mouse strains throughout the infection (N. Starobinas *et al.*; S.M.D. Moraes *et al.*, unpublished data), although normal macrophages from the resistant strain produced higher levels of this cytokine than those from the susceptible strain after their *in vitro* cocultivation (M.S.M.V. Sarti and V.L.G. Calich, unpublished data). TGFβ was produced only at the onset of the i.p. infection, although higher levels were produced by macrophages from the susceptible strain (Moraes *et al.*, 1997). These monokines, in concert with the effects of other cell populations and products of innate immunity, may influence the patterns of granulomas as well as the type of the acquired immune response developed after *P. brasiliensis* infection.

B lymphocytes and their products in the isogenic murine model of PCM

In PCM, the production of high levels of specific antibodies is not protective. In fact, such a behaviour is long known to represent a bad prognosis of the disease (Fava Netto, 1955; Biagioni *et al.*, 1984). The specific antibody production was analysed in the course of i.p. and i.t. infection of susceptible and resistant mice. It was found that independently of the route of inoculation, the production of total specific antibodies by the susceptible mice was higher and more precocious than that of resistant ones. The levels of IgM- and IgG-specific isotypes were also constantly higher in the susceptible mice, indicating a more pronounced tendency towards development of the humoral immunity in those animals (Calich *et al.*, 1988; Vaz *et al.*, 1992; Cano *et al.*, 1995). The antibody repertoire produced by A/Sn and by B10.A mice was analysed by immunoblotting and was found not to substantially differ, demonstrating that susceptibility and resistance were not governed by the differential recognition of some antigenic components of *P. brasiliensis* (Vaz *et al.*, 1992). Both strains synthesized specific antibodies of all isotypes, but their levels and the kinetics of their production varied. Susceptible animals produced higher levels of IgA (i.p.) and IgG2b (i.p. and i.t.) and resistant mice, higher levels of IgG3 (i.t.) and IgG2a (i.p. and i.t.) isotypes (Calich *et al.*, 1995; Cano *et al.*, 1995). Therefore, the susceptible mouse strain produced higher levels of isotypes elicited by type 2 and inhibitory cytokines such as IL5 and TGFβ, whereas the resistant strain synthesized specific antibodies mainly regulated by IFNγ.

The severe cases of PCM show polyclonal activation of B lymphocytes (Chequer-Bou-Habib *et al.*, 1989), in which a large number of plasmocytes secrete irrelevant antibodies. In the murine model, the resistant animals showed a small increase in the number of B lymphocytes secreting IgM and IgG3, whereas the susceptible mice showed evident polyclonal activation of IgG1-, IgG2b- and IgG2a-producing B cells (Calich *et al.*, 1994). These findings in the human and experimental diseases confirm the preferential activation of humoral immunity in the severe forms of PCM.

T lymphocytes and their products in the isogenic murine model of PCM

The important role played by cellular immunity in PCM (Mok and Greer, 1977) led us to evaluate the specific delayed-type hypersensitivity (DTH) reaction in the course of i.p. infection. In fact, the results obtained were similar to those observed in patients, in that the healing mice developed and maintained fungal-specific DTH responses, whereas the reactions in susceptible mice, although beginning already at the second week of infection, soon subsided, being followed by constant anergy. This anergy was *P. brasiliensis*-specific, as could be concluded by the normal responses developed by infected animals towards unrelated antigens. The positive reactions detected at the beginning of the infection showed that the anergy is not due to an inherent inability of the susceptible mice to mount DTH responses to *P. brasiliensis* antigens, but to their genetic predisposition to develop immune responses which result in impaired cellular immunity (Fazioli *et al.*, 1994). *In vivo* experiments of depletion of the T $CD4^+$ and T $CD8^+$ cells demonstrated that both cell subsets are involved in the DTH responses of susceptible animals, whereas in resistant mice such responses are entirely mediated by T $CD4^+$ cells (R.A. Fazioli and V.L.G. Calich, unpublished results). Thus, the different patterns of DTH responses developed by susceptible and resistant mice reflect the fact that they were originated from interactions among distinct cell populations, which certainly react in diverse ways to the antigenic stimulus of *P. brasiliensis*.

The cellular immune response was also studied *in vitro*, analysing the proliferative responses of lymph node cells to *P. brasiliensis* antigens in the course of i.p. infection. In both B10.A and A/Sn mouse strains, the intensity of the proliferative response was always low when compared to that of animals presenting a healing infection induced by the subcutaneous (s.c.) inoculation, suggesting the existence of inhibitory phenomena related to the severity of the disease. Indeed, anti-TGFβ monoclonal antibodies (mAbs) reversed the low levels of proliferative responses of both strains, but this effect was much more prominent in the lymphocytes from the susceptible strain. The kinetics of the lymphoproliferative response differs in the two strains, in that susceptible mice present significant proliferation already at the second week of i.p. infection, whereas

the resistant ones do so only at later stages. Furthermore, analogously to what was observed in the DTH reaction, lymphoproliferative responses of susceptible mice were inhibited by *in vitro* treatment with anti-T $CD4^+$ and anti-T $CD8^+$ mAb, whereas that of resistant mice only by anti-T $CD4^+$ mAb (Fazioli *et al.*, 1997). The importance of the body compartment in which the immune response takes place is illustrated by the different behaviour of lymphocytes from the spleen, stimulated by *in vitro*-pulsed normal APC, where lymphocytes from the resistant strain maintained their ability to proliferate and those from the susceptible strain gradually became anergic (Sbravate *et al.*, 1995). Therefore, analogously to what was observed in the DTH reactions *in vivo,* susceptibility seems to be associated with precocious stimulation of T cells followed by anergy.

In the lymph nodes at the site of i.p. infection, an intense and early increase in the number of T lymphocytes of the $TCR\alpha\beta^+$, $CD4^+$, $CD8^+$ and $TCR\gamma\delta^+$ phenotypes as well as of B lymphocytes was detected in the susceptible mice, as compared to a discrete and late increase in these cellular subpopulations in the resistant mice. In the regional lymph nodes of susceptible mice, a striking increase in the absolute numbers of activated T $CD8^+$ and B lymphocytes, as detected by the concomitant presence of the IL2 receptor, occurred during the whole course of the infection, in contrast to the late increase detected in the resistant animals (R.A. Fazioli and V.L.G. Calich, unpublished results). Thus, susceptibility is marked by intense cellular multiplication and activation in response to *P. brasiliensis* infection, suggesting that excessive mobilization of the immune system at very early phases of PCM is deleterious to the development of protective immunity. Furthermore, the apparent anergy of these mice is associated with an intense cellular multiplication in the regional lymph nodes.

Following restimulation with *P. brasiliensis* antigen, total lymph node cells from i.p.-infected resistant mice produced early and sustained levels of IFNγ and IL2, whereas susceptible animals secreted low to undetectable quantities of these type 1 lymphokines. Both mouse strains present late and transient production of IL4, while IL10, constantly produced throughout the disease, was secreted in higher levels by susceptible animals. IL5 was produced by resistant mice in the chronic phase of the infection, whereas susceptible mice had two peaks of IL5 production, at the first and 12th weeks postinfection. Only the susceptible mice presented medullary and splenic eosinophilia concomitant with raised IL5 production (Calich and Kashino, 1998; Meloni-Bruneri *et al.*, 1994).

The synthesis of lymphokines was also analysed one and two months after i.t. infection. Although susceptible and resistant mice produced IFNγ, IL2, IL4, IL5 and IL10 consistently, all these lymphokines, except IL2, were produced in higher levels in the susceptible mice. Again the presence of IFNγ in a context of disease control is patent, because at the beginning of pulmonary infection, susceptible mice are able to control *P. brasiliensis* in the lungs (Cano *et al.*, 1998).

The dichotomy of type 1 and type 2 T $CD4^+$ lymphocytes secreting different lymphokines which preferentially enhance cellular or humoral immune responses has greatly helped to better understand the immunological mechanisms involved in the control of several infectious diseases. The knowledge gathered analysing human and murine PCM strongly suggested that this kind of immunoregulation would occur in this disease (Musatti *et al.*, 1994; Calich *et al.*, 1994). However, in the present state of knowledge, it is essential to analyse the events occurring in an infected host considering that the types and levels of different cytokines vary in the course of the infection, as well as in the various body compartments. Their effect as a whole is the overall result of numerous synergic or antagonistic interactions which occur after a polyclonal immune response due to the numerous immunogenic epitopes present in the parasite. For instance, IL5 was able to induce eosinophilia in the context of type 2 cytokines, as occurs in most infectious processes, inclusive in susceptible mice infected with *P. brasiliensis.* However, IL5 was unable to elicit a similar phenomenon in the presence of type 1 lymphokines, as was the case in resistant mice. The inhibitory effect of IL10 could prove to be deleterious to the susceptible mice, but useful to the resistant ones probably by buffering excessive inflammatory responses.

PCM in immunodeficient and immunomanipulated animals

PCM in athymic mice

Murine PCM was comparatively studied using athymic (BALB/c, nu/nu) animals, which have a severe deficiency of immune responses mediated by T lymphocytes, and their euthymic (BALB/c, nu/+) T cell normal counterparts. The nude (nu/nu) animals infected with the virulent (Pb 18) *P. brasiliensis* isolate presented a shorter survival time and a more severe disease when compared with euthymic mice. Histopathological studies revealed that the main structural difference between lesions developed by nu/+ and nude mice resided in the more encapsulating tendency in the former ones. At the first week after infection, there was evidence of fungal destruction and control of its proliferation in both animal groups. From week 4 on, only nu/+ mice maintained the control of infection, while nu/nu mice showed a tumour-like progression of the disease with high

numbers of infected organs (Burger *et al.*, 1996a). These results confirm a previous report (Miyaji and Nishimura, 1983), demonstrating the important role played by T cells in the host defences to *P. brasiliensis* infection, but also suggest that mechanisms of innate immunity are equally important in the initial control of infection. Further evidence of the protective effect of natural immunity came from the resistant behaviour of athymic animals which did not develop a progressive disease when infected with a slightly virulent (Pb 265) *P. brasiliensis* isolate (Burger *et al.*, 1996b).

Using the same animal model, and analysing the humoral immune response by immunoblotting, we were able to define the T-dependence and T-independence of *P. brasiliensis* antigens (Burger *et al.*, 1996b). Thus, the majority of *P. brasiliensis* components are T-dependent, but the immunodominant gp-43 and also the 41.5 and 27.5-kDa antigens were characterized as T-independent components. It is tempting to speculate that these molecules could play a role in the escape mechanisms evolutionarily developed by *P. brasiliensis* through its ability to directly activate B cells, giving rise to fungi-specific APC which preferentially drive the immune response to a non-protective Th2 pattern (Fitch *et al.*, 1993; Mason, 1996).

PCM in CD8$^+$ and CD4$^+$ T-cell-depleted mice

We have also examined the contribution of CD8$^+$ T cells in the course of pulmonary PCM (L.E. Cano and V.L.G. Calich, manuscript in preparation). *In vivo* treatment with anti-CD8$^+$ mAb caused impaired host defences of both mouse strains. Depletion of CD8$^+$ T cells of A/Sn mice in 4 weeks did not alter the degree of the infection, suggesting that CD8 cells do not play an important role at the initial phases of the disease of resistant animals. However, these cells appear to be involved in the later control of the pulmonary infection, hampering the escape of fungal cells to other organs, since prolonged times of depletion (8 weeks) resulted in extrapulmonary fungal dissemination, although the pulmonary infection remained unaffected. In susceptible mice, however, CD8$^+$ T cells seem to have a protective role at the beginning of the infection, since at the 4th week postinfection, CD8$^+$-cell-depleted mice presented an exacerbated pulmonary infection accompanied by a precocious fungal dissemination to the liver and spleen; this profile was maintained after prolonged times of depletion.

Ablation of CD8$^+$ T cells did not alter DTH reactions of A/Sn mice, but increased these reactions in B10.A mice. Thus, in susceptible mice, protection and DTH reactions are dissociated traits and it appears that CD8$^+$ T cells may be involved in both protection against *P. brasiliensis* and the negative regulation of DTH responses. The production of *P. brasiliensis*-specific antibodies by CD8$^+$-depleted resistant and susceptible mice was similar to that of mice given control antibody. Taken together, these results indicate that CD8$^+$ T cells are necessary for optimal clearance of the fungus from tissues of mice infected with *P. brasiliensis*, and demonstrate an earlier and more prominent protective activity of this T-cell subset in the immune response mounted by susceptible animals. Moreover, the experiments performed in CD8$^+$-depleted animals indicate that *P. brasiliensis* antigens probably can be presented by MHC class I molecules despite the preferential endosomal behaviour of yeast cells (Brummer *et al.*, 1989). Our results also indicate that, early in the disease, the antigen processing by APC of susceptible animals is different from that of resistant mice. In B10.A mice, *P. brasiliensis* antigens appear to gain access into MHC class I molecules and vigorously activate protective CD8$^+$ T cells. In addition, depletion of CD8$^+$ T cells in the i.p. model of infection confirmed the participation of these cells in the DTH reactions of susceptible but not of resistant animals as well as their minor contribution to the regulation of antibody production by both mouse strains. The influence of CD8$^+$ T cells on DTH reactions was also observed in studies with other fungal diseases. Depletion of CD8$^+$ cells during experimental cryptococcosis is associated with suppression of specific DTH responses (Mody *et al.*, 1993), whereas in histoplasmosis, depletion of these cells does not alter the onset or maintenance of DTH reactions (Deepe, 1994).

We have also carried out some studies on the participation of CD4$^+$ T cells in the immune response mounted by A/Sn and B10.A mice after *P. brasiliensis* infection (L.E. Cano, S.S. Kashino, R.A. Fazioli and V.L.G. Calich, unpublished observations). Although performed with a less virulent isolate, depletion experiments of CD4$^+$ T cells demonstrated that this cell subpopulation is not necessary to control pulmonary infections caused by isolates of low virulence. Elimination of CD4$^+$ cells significantly reduced the DTH reactions and practically abrogated the antibody production by resistant and susceptible mice infected by either the i.t. or i.p. routes. These results, although needing further studies with a more virulent isolate, demonstrated that CD4$^+$ T cells play a dominant role in the cellular and humoral immune responses induced by *P. brasiliensis* infection.

PCM in IFNγ-depleted mice

Clinical studies (Bava *et al.*, 1991; Bernard *et al.*, 1995), as well as the immunological phenotype displayed by animals of polarized behaviour of our experimental model (Calich *et al.*, 1994; Cano *et al.*,

1995), suggested that resistance to PCM was associated with immune responses governed by IFNγ. As already discussed, the i.p. infection of resistant animals was associated with a sustained production of IFNγ, and the unexpected superior ability of susceptible animals to control fungal growth in the lungs after i.t. infection was also accompanied by the presence of high levels of pulmonary IFNγ (Cano *et al.*, 1998). To better understand the biological effects of this cytokine, we investigated the effect of endogenous IFNγ depletion by mAb in the course of the i.t. infection of susceptible and resistant mice. In both mouse strains, neutralization of IFNγ induced exacerbation of pulmonary infection, earlier fungal dissemination to liver and spleen, impairment of specific DTH responses and increased levels of IgG1 and IgG2b antibodies. Depletion of IFNγ increased more than 1,000-fold the pulmonary fungal burden of B10.A mice and 10-fold the already high number of fungal cells in the lungs of A/Sn mice, which became indistinguishable from those in susceptible animals. This finding clearly showed the critical role played by IFNγ in host resistance against *P. brasiliensis* infection. Moreover, *in vivo* neutralization of IFNγ dramatically altered the histological pattern of pulmonary lesions of B10.A and A/Sn mice, which lose their ability to circumscribe fungal cells at the site of infection. The compact granulomas composed of packed epithelioid cells and a restricted number of fungi were replaced by a fungus-rich diffuse inflammation obliterating the normal pulmonary structure.

PCM in IL4-depleted mice

To better understand the immunoregulatory mechanisms involved in susceptibility to pulmonary PCM, B10.A animals were *in vivo* depleted of IL4, a cytokine which favours Th2 development (Reiner and Lockesley, 1995; Seder and Paul, 1994). The depletion of IL4 by mAb (1 mg i.p./week) did not alter the susceptibility pattern of B10.A mice which presented the same severe disseminated disease as their untreated infected counterparts. In addition, IL4 neutralization did not change the predominant secretion of type 2 cytokines in the lungs, and ephemerally lowered the levels of IL10 present in the spleen. Also, no significant differences were noted in the pulmonary histopathological features (Arruda *et al.*, 1997). These findings suggest that IL4 does not play a prominent role in host susceptibility to *P. brasiliensis* infection. This conclusion is different from those obtained in polarized Th1/Th2 models of infection employing several microrganisms, in which abrogation of endogenous IL4 leads susceptible hosts to a healing phenotype (Romani *et al.*, 1992; Sadick *et al.*, 1990). In another experimental model of pulmonary PCM using BALB/c mice, Hostetler *et al.* (1993) demonstrated that early *in vivo* treatment with 8 mg of anti-IL4/mouse had a beneficial effect on the disease. Since we used much lower doses of mAb, other protocols using higher amounts of anti-IL4 should be tested in our experimental model.

Effect of recombinant IL12 (rIL12) administration

The critical role played by IFNγ in the resistance mechanisms against *P. brasiliensis* infection (Cano *et al.*, 1998) and the well-described ability of IL12 to stimulate IFNγ production by T lymphocytes and NK cells (reviewed by Trinchieri, 1995) led us to investigate the effect of early administration of exogenous IL12 in the pulmonary disease developed by susceptible animals (Arruda *et al.*, 1997). Thus, rIL12 (a generous gift of S. Wolf, Genetics Institute, Boston) was administered i.p. (1 μg/mouse/day for 5 days, starting at day 0 after infection) to susceptible B10.A mice which were tested at weeks 4 and 8 after i.t. infection. This treatment markedly inhibited yeast dissemination to liver and spleen (more than 100-fold), although the pulmonary fungal load and DTH reactions remained unchanged. Histopathological studies, however, demonstrated a dramatic increase in the mononuclear cell infiltration present in the lungs of IL12-treated B10.A animals. This finding is consistent with a pulmonary model of *Cryptococcus neoformans* infection where the protective activity of IL12 was accompanied by a marked infiltration of inflammatory mononuclear cells in the lungs and impaired dissemination of the fungus to the brain, resulting in the prolonged survival rate of infected mice (Kawakami *et al.*, 1996).

In murine PCM, the protective effect of IL12 was detected at week 8 after infection and was associated with decreased levels of type 2 cytokines (IL4 and IL10) present in the lungs. Unexpectedly, the pulmonary levels of type 1 cytokines (IL2 and IFNγ) were also diminished at the same postinfection period. Over the past few years, it has become clear that IL12 is the principal cytokine in inducing Th1 responses through its ability to elicit IFNγ production by T lymphocytes and NK cells (Trinchieri, 1995). In our experimental protocol, however, increased production of IFNγ was not observed after 4 and 8 weeks of infection, but measurements of cytokines at earlier periods of infection will probably help to elucidate this issue. Treatment with rIL12 abrogated the synthesis of IL10 and induced a two-fold increase in the proliferative response of lymph node cells following stimulation with a soluble *P. brasiliensis* antigen. In parallel with the lowered levels of pulmonary IL4, a diminished production of IgG1-specific antibodies was observed in IL12-treated mice. Our findings suggest that the protective effect of IL12 is more likely exerted through

the decreased production of type 2 cytokines (IL4 and IL10) than through the sustained production of augmented levels of IFNγ. A similar finding was reported for mice systemically infected with *C. albicans* showing that IFNγ production occurs in mice with either the healing or the non-healing phenotype, and Th1 development correlates with IL12 but not IFNγ production (Romani *et al.*, 1994). In support of these observations, results from our laboratory have also associated protective immune responses in the pulmonary PCM of B10.A mice with increased production of IL12 (C. Arruda and V.L.G. Calich, unpublished observations). The ability of rIL12 to improve PCM in innately susceptible B10.A mice strongly supports a role of this cytokine in the induction of protective immunity to *P. brasiliensis* infection. In conclusion, to our knowledge this is the first demonstration of the therapeutic effect of IL12 in murine PCM, suggesting that protective immune responses can be induced in susceptible hosts.

Protection and exacerbation models of PCM

The s.c. route of inoculation stimulates suitable cellular and humoral immune responses and it has been employed to induce protective effects to some pathogenic fungi such as *C. neoformans* (Moser *et al.*, 1982) and *Blastomyces dermatitidis* (Spencer and Cozard, 1973).

Both susceptible and resistant mice inoculated with *P. brasiliensis* by the s.c. route developed a very mild infection, characterized by the absence of lesions in all organs/tissues examined. Despite the equivalent self-healing disease outcome, B10.A animals presented significantly higher DTH responses than those of A/Sn mice. Furthermore, B10.A animals developed DTH reactions with fungal infecting doses 100-fold lower than those required to prime A/Sn animals (5×10^4 yeast cells for B10.A and 5×10^6 yeast cells for A/Sn). Interestingly, B10.A mice which were anergic to specific DTH reactions, in the i.p., i.t. and intravenous (i.v.) models, developed more intense DTH reactions than their resistant counterparts. The higher DTH reactivity of B10.A mice was associated with a high production of IgA-, IgG1-, IgG3- and IgG2b-specific antibodies, indicating a concomitant activation of Th1 and Th2 cells (Arruda, C. *et al.*, 1994). Our results clearly show that the immunoregulatory mechanisms, which account for the lack of stable cell-mediated immunity in susceptible animals after i.p., i.v. and i.t. routes of infection, are absent or less evident in the infection induced by the s.c. route.

Since B10.A mice do not present any intrinsic deficiency in the ability to develop an effective cellular immune response, a protective immunization protocol was developed inoculating those animals with five million *P. brasiliensis* yeast cells by the s.c. route. At the optimal time for development of a DTH response (2 weeks after infection), mice were challenged i.p. or i.v. with a lethal dose of the same isolate. The i.p.-challenged animals survived significantly longer than the non-vaccinated controls. Surprisingly, the animals challenged i.v. presented increased mortality compared to the iv infected controls. These results showed that the same immune response developed following s.c. fungi inoculation led to opposite disease outcomes, depending on the route of challenge (Arruda *et al.*, 1994).

This protection/exacerbation model of PCM was further explored. The lower mortality observed after s.c. priming and i.p. challenge correlated with lower fungal loads in several organs, sustained DTH responses (reversal of the anergy induced by the i.p. route), higher lymphoproliferative responses, increased production of specific antibodies (IgM, IgG1 and IgG3) and enhanced production of IL2 and IL10 by antigen-stimulated lymph node cells. In conclusion, the protection model showed that B10.A animals can indeed develop a protective immunity which is associated with secretion of higher levels of IL2 and IL10 and the reversal of DTH anergy, turning those susceptible mice into resistant ones. This model of PCM indicates that a balanced type 1/type 2 immune response is sufficient to confer protection to susceptible hosts (Arruda *et al.*, 1994; Calich and Kashino, 1998).

The exacerbated disease induced by i.v. challenge was further studied in B10.A mice. Despite the higher mortality, the s.c.+i.v. treated mice did not show an increased fungal load in the examined organs. However, those animals were persistently anergic in terms of DTH reactions (like their i.v. infected counterparts), showed suppressed IL2 production, presented higher levels of IgM and IgA specific antibodies and decreased levels of IFNγ-induced specific isotypes (IgG2a), and secreted enhanced levels of IL4, IL5 and IL10. In this model of exacerbated disease, the association of DTH anergy with enhanced secretion of IL4, IL5 and IL10 points out the deleterious effects of type 2 cytokines (Calich and Kashino, 1998).

Data from the literature have suggested that the type of APC, as well as the differences in the densities of ligands and costimulatory molecules, determine the phenotype of responding T cells (Gajewski *et al.*, 1991; Fitch *et al.*, 1993; Kuchroo *et al.*, 1995). Purified splenic B cells stimulate optimal proliferation of Th2 cells while adherent spleen cells stimulate optimal proliferation of Th1 cells. A functional dichotomy of APC was also found by Schmitz *et al.* (1993), who suggested that, *in vivo*, initial dominant presentation of antigen by macro-

phages induces cell-mediated immunity and initial antigen presentation by B cells induces humoral immunity. Thus, the site of the immune response and the route of entry of antigen could play a critical role for selection of effector functions of the immune response. Along this line, different patterns of immune response to *P. brasiliensis* after inoculation by diverse routes could be the result of distinct APC interacting with T cells leading to the preferential activation of type 1 or type 2 subpopulations and consequent polarized immune responses. One can speculate that in the protection model of PCM (s.c.+i.p. routes), Langerhans cells, dendritic cells and peritoneal macrophages could be the main APC which may favour a more balanced activation of type 1 and type 2 cells resulting in preserved cell-mediated immunity which leads to a benign disease and self-healing; in the exacerbation model (s.c.+i.v. routes), although the presence of Langerhans cells is found at the initial phase, after i.v. challenge, the splenic B cells could dominate the activation pathways and play a critical role favouring the development of type 2 subpopulations and humoral immunity, leading to severe disease. Although it is very attractive, this hypothesis does not clearly explain the differences in responses found in susceptible and resistant mice after sole i.p., i.t. or i.v. infection. Nevertheless, since susceptible mice were found to be much more reactive to *P. brasiliensis* infection and present, in regional lymph nodes, a 3-fold increase in the number of B cells, a fact not observed in resistant mice, it is conceivable that this increased population of B cells acquires the role of the main APCs of the response. Indeed, previous results showed that depletion of B cells in B10.A mice infected i.p. with *P. brasiliensis* causes a milder disease than that of infected controls, presenting a markedly different fungal load (much lower in the lungs and spleen and no yeast cells in the liver), favouring the hypothesis that hyperactivation of B cells may lead to the deleterious effects of the immune response (L.G. Mendes Jr., V.L.G. Calich and C.A.C. Vaz, unpublished data).

Concluding remarks

Our experimental murine model appears to mimic human PCM, with resistant mice reproducing the mild forms and susceptible mice the severe chronic disease. The studies employing these mouse strains with polarized behaviour, submitted to infection by several routes and to immunomanipulations, enabled us to improve our knowledge of the host-parasite relationship in this mycosis. The main conclusions or inferences so far obtained are:

a) Resistance to *P. brasiliensis* infection is associated with immune responses which favour cellular immunity and activation of phagocytes throughout the infection, whereas premature deactivation of T-cell-mediated immunity and activation of B cells lead to severe disease outcome.

b) Polyclonal activation of B cells as well as production of high levels of specific antibodies are indicative of progressive disease. However, the production of certain immunoglobulin isotypes such as IgG2a correlates with protective immunity. In addition, most *P. brasiliensis* antigens are T-dependent, but some fungal components can directly activate B cells.

c) Resistant and susceptible mice do not develop a totally polarized Th1/Th2 pattern of immune response. However, secretion of IL12 and IFNγ is protective, while the dominance or early secretion of IL10 or TGFβ is associated with susceptibility.

d) Resistance is associated with an equilibrated and progressive activation of T $CD4^+$ and B cells, whereas susceptibility parallels a more intense and precocious activation of T $CD4^+$, T $CD8^+$ and B cells. In addition, there is evidence that T-cell activation occurs by both class-I and class-II-restricted pathways.

e) T-cell-deficient mice retain the ability to regulate expansion of the fungal population during the early phases of infection, indicating an important role for innate immunity in PCM. Moreover, the presence of IL12 early in the infection determines less severe patterns of disease.

f) The cytokine milieu, as well as the diverse types of APC present in different organs or tissues, have a profound influence on the immune response and determine different disease outcomes.

g) Usually, strong DTH reactions are associated with protective immune responses; however, this reaction is not *per se* prognostic for resistance.

h) Several parameters indicate that susceptible animals are more reactive to *P. brasiliensis* infection, and downregulatory mechanisms of cellular immunity appear to be linked to the non-protective immune responses.

i) Susceptibility can be circumvented by immunointervention, for instance, by the administration of IL12 or by previous s.c. infection.

In conclusion, we believe that the isogenic murine model of PCM provided new instruments for a better comprehension of the immunopathogenesis of this deep mycosis. Furthermore, the increasing knowledge of the immunological mechanisms which confer resistance to PCM, as well as to other mycotic infections, opens new perspectives for the design of novel prophylactic and therapeutic strategies.

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Protective immunity in coccidioidomycosis

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The life cycle and biology of *Coccidioides immitis*

Coccidioides immitis is classified as a dimorphic fungus, but actually assaults the host with a multifarious range of fungal morphotypes (Cole and Sun, 1985). Infection is initiated by the airborne arthroconidia; a desiccated, disarticulated mycelial fragment which measures in 2-4 μ in size. In the lungs of the host, the arthroconidium begins to round up and enlarge, becoming a young spherule. This begins the parasitic cycle where the spherule matures and forms internal compartments by segmentation which give rise to endospores. At maturity, the spherule ranges from 60-80 μ in size and ruptures, releasing endospores in large packets encased in a fibrillar matrix. Individual endospores, measuring 3-5 μ in diameter, can break away from the packet and each one can go on to form a new spherule and renew the parasitic cycle with a regeneration time of approximately three days. Thus, the host must contend with a wide range of organism sizes from the arthroconidium and endospore, which are small enough to be phagocytized by polymorphonuclear leukocytes (PMNL) and macrophages/monocytes, to the mature spherules which are eight to ten times the size of phagocytes (Sun *et al.*, 1986). If the endospores are released into the environment, they convert into the mycelial phase or saprobic cycle in the soil. It is believed that mycelia usually grow within the first 12 inches of soil during optimum growth conditions and, as the growth conditions deteriorate, alternating cells degenerate leaving the remaining viable cells to form arthroconidia. When the soil is disturbed, arthroconidia break away from the degenerating mycelia and become airborne. Carried aloft by prevailing winds, arthroconidia are then available for inhalation by a potential host.

C. immitis is endemic in the arid southwestern United States and Mexico in the geographic area termed the Lower Sonoran Life Zone which also extends into parts of Central and South America

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